

Aftermath of the Mark VA

THE tale of the Jodrell Bank Mark VA radiotelescope that was never built is a gloomy one from any point of view. The Fifth Report from the Commons Committee of Public Accounts (HMSO; £3.45) takes a look at it.

Original plans were for the Science Research Council (SRC) to construct a 400-foot steerable dish (the Mark V); the council sought approval from the Department of Education and Science (DES) in 1970 on the basis of an estimate of £6.2 million (January 1970). A revision of the cost to £8 million caused the SRC to propose a smaller telescope—the Mark VA, with a 375-foot aperture, which they hoped could be built within the £6.2 million figure. A detailed design was to be prepared by the consulting engineers Husband and Company (a firm well experienced in radiotelescopes), covering drawings, bills of quantities and an invitation to tender for construction. The United Kingdom Atomic Energy Authority (UKAEA) would act as project managers, and it was estimated that £245,000 would be needed in consultants' fees. A large fraction of this figure was tied to the final tender price for construction.

Treasury approval for the design stage was given in June 1971. Ten months later the consultants were given the go-ahead to complete the design, if possible, within twelve months. They were told to alert the UKAEA if it seemed that the construction would cost more than was originally planned (due allowance had been made for inflation). Within six months they reported that the limit had been reached. After that, however, they were under no obligation to report costs further, and indeed had declined to have any further reporting written into their contract. The SRC's explanation for this was that "the consulting engineer . . . was designing the telescope to make it as cheap to build as he could. He was still finding out how it could be built cheaply, and it was more important for him to do that than to keep estimating what the cost was".

The inevitable happened. In October 1973 the consultants finished; tenders received in March 1974, reflecting the great uncertainties then prevailing in the construction industry, were around the £14 million mark. The total cost was put at £16 to £17 million. (It had been estimated just before by the UKAEA that the £6.2 million—at 1970 prices—which was the original total cost would have inflated to £10.7 million by January 1974.) Faced with a 50% growth in real terms in costs, and having to live with negligible overall growth, the SRC abandoned the telescope in June 1974, having

learnt that total costs had risen even more in the interim to £22 million.

But this left one matter outstanding—the consultants' fee. As it was related to the tender price, it now amounted to the sum of almost £600,000, although on this the consultants eventually offered a rebate of £30,000. Even so, the amount they received was rather more than double what they must have expected on starting—a tidy windfall even when inflation had taken its toll. The SRC, which had not been informed itself of price escalation, had in its turn not been able in 1973 to keep the DES and the Treasury alerted to rising real costs, and had to seek special Treasury authority for the total design bill of £660,000.

It is fairly easy now to look back and see what went wrong. People were unused to dealing with inflationary conditions, and so didn't exercise such stringent controls as they would now. Even so, the telescope might have come through unscathed were it not for the situation which developed nationally during precisely the months that potential contractors were preparing their tenders. As it is the SRC gets a mild rap over the knuckles for letting matters drift, and rightly so, although the report has a rather optimistic view of the willingness of the scientific community to drop major projects when costs escalate. But what about the method by which the consultants were paid?

It must be made clear that the issue here is in no sense one of indulging in any gold-plating. The figures mentioned and the evidence of the SRC and the DES all fit in with the appalling way in which construction costs escalated. Be that as it may, the taxpayer ended up paying the consultants an "uncovenanted benefit", in the words of the committee, totally unrelated to the cost of the project. What is more, the SRC, having decided not to go ahead, would have to pay an additional fee if it chose to think again, as the plans are the copyright of the consultants. The SRC did at the very outset enquire about retaining the drawings, but was told that this would roughly double the fees.

All of this is legal and common practice. Fees are related to tender price, and copyright stays with the designer. But it does seem to the outsider to be open to charges of being inequitable, and it can lead to cynicism. The Public Accounts Committee calls for further consideration to be given to the way consultancy agreements work in the public sector. We hope someone is doing just that. □



Energy from warm rocks

Ron Oxburgh examines the prospects for geothermal energy in Britain, where a new research programme is to begin

IN the first few years of this century, an electrical generator with an output of several kilowatts was installed at Lardarello in northern Italy, to be driven by the steam which emanated naturally from the ground. It was the first modern attempt to harness the natural heat of the Earth to man's purposes. Relatively little further progress was made until the 1930s, but since then the use of geothermal energy has increased considerably, until today about 1100 MW are generated by this means in seven countries. Most of these installations pre-dated the rapid rise in fossil fuel prices of the past few years, and even then were competitive with conventional means of power generation. Today their situation is still more favourable.

Fifty or more countries are now involved in geothermal exploration. The number was recently increased by one with the decision announced by the UK Department of Energy to spend £840,000 on the geothermal exploration of Britain over the next three years. That decision was made largely on the basis of Energy Paper No. 9 (*Geothermal energy: the case for research in the United Kingdom*), a special report prepared by the Energy Technology Support Unit at Harwell, in which Dr John Garnish, the author, argues cogently for a cautiously optimistic approach to the prospects of geothermal energy in the UK.

Heat by convection

Heat is continuously generated within the Earth by the radioactive decay of unstable isotopes (mainly of U, Th and K) and is lost from the surface at an average rate of about $1.5 \mu\text{cal cm}^{-2} \text{ s}^{-1}$ (63 mW m^{-2}). At depths greater than about 80 km heat transfer within the Earth is mainly by convection, but above that mainly by conduction; rocks are relatively poor conductors of heat and within this outer zone the conductive thermal gradient ranges from less than 10°C km^{-1} to about 60°C km^{-1} . This is true over more than 95% of the Earth's surface, within the interiors of the great tectonic plates.

Along the margins of the plates, however, and very occasionally within them, heat may locally be transferred to the surface or to within a few kilometres of it by convection. The convective medium in these cases is magma, or molten rock, at temperatures between 800°C and 1100°C . The magma commonly interacts with ground water circulating in the pores

of the near surface rocks to give rise to geysers, hot springs or fumaroles. Drilling to 1,000 m or so in such areas may provide flows of water or steam at between 200°C and 300°C which, in favourable circumstances, can be used to drive turbines and generate electricity. All the major producers of geothermal electricity—Italy, the Western USA, New Zealand, Japan and Mexico (1,030 MW together)—exploit situations of this kind, and are all situated in the tectonically unstable earthquake and eruption-prone margins of plates.

Rocks at comparable temperatures may be found beneath other parts of the Earth's surface, such as the UK, where subsurface temperatures are governed largely by conduction, but they are much deeper. At the depth limit of present drilling experience ($\sim 9 \text{ km}$ for a cost of £2–3 million), temperatures in such areas rarely exceed 300°C . Furthermore, it is not sufficient to reach hot rock; if the heat is to be used it must be extracted by means of circulating fluids, and for this process to be effective the fluid must be able to permeate the hot medium thoroughly through pores and fissures. With increasing depth, however, the weight of the overlying rock tends to close pores and fissures and reduce the permeability to a very low value.

For these reasons, in most places the hot rocks which everywhere underlie us are at present both too expensive to reach and too impermeable to exploit, and are rather unlikely to provide the large quantities of water suitable for the economic generation of electricity by conventional technology (temperatures in excess of 200°C are required).

Alternative applications

If, however, we consider alternative applications of geothermal energy, the position is quite different. There is a wide range of uses for water above 65°C when it is used directly in a heat exchanger rather than to generate electricity. Taking 100°C as a convenient reference value, rocks at this temperature may be reached virtually anywhere in the world by drilling holes well within the depth range of present technology. Whether these "warm rocks" constitute an exploitable natural resource depends upon the drilling costs of reaching them, their permeability structure, and the availability of a local demand for the low grade heat they can supply.

The economic considerations are now more finely balanced. As a very rough indication: given both suitable reservoir conditions at depth, and a suitable local market for the hot water, warm rock geothermal energy is at present competitive with fossil fuels if temperatures of 100°C can be reached at 3 km or less. If, however, the cost of fossil fuels rises faster than drilling costs, this depth will be increased and the position of geothermal resources relatively improved.

Viewed in this way, the evaluation of UK geothermal resources becomes a problem of identifying those areas with slightly higher than average geothermal gradient and of understanding the subsurface distribution of permeability sufficiently well to distinguish those which are capable of successful exploitation. This latter problem is not a simple one, but is tractable by combining the expertise of the oil industry in understanding and controlling the flow of fluids underground with experience of hydrogeologists of rock permeabilities and natural underground circulations of water in the UK.

The problem of identifying regions of sufficiently high temperature is less easy. In the UK these are unlikely to be so pronounced that they give rise to significant perturbations of the surface magnetic field or the electrical conductivity structure of the crust, and there is little alternative to some kind of direct measurement of temperatures. Broadly, two approaches are possible. Deep holes may be drilled and temperatures measured at the proposed depth of exploitation. This is undoubtedly the safest method of exploration, but it is impossibly expensive unless the hole has been drilled for some other purpose which meets most of the costs.

The other approach is to make very precise measurements in shallow holes and use these as a basis for extrapolation to greater depth. The difficulty here is that near surface temperatures may be influenced by climatic changes (ice age effects may still be recognised hundreds of metres below the surface), the circulation of ground water, topographic irregularities of the surface and even erosional processes; for these reasons measurements are made in holes more than 200 m deep and preferably more than twice that depth, and are then corrected for the effects of the various perturbing factors. The heat flux, q , is given by $q=k\beta$ where k is the mean thermal conductivity of the rock, and β the mean thermal gradient, over a particular depth interval. The heat flow value determined in this way may then be used to predict the temperature at some greater depth with an accuracy which depends largely upon how well the thermal conductivity of the rocks down to that depth is

known. Heat flow measurements of this kind must form the main basis of any exploration programme.

Although a relatively large number of underground temperatures have been measured in the UK, there have been relatively few reliable heat flow measurements—about 25. Although many of the temperature measurements were sufficiently accurate for the purposes for which they were made (ventilation in mines, for example), they do not form a reliable basis for extrapolation to significantly greater depths. Similarly the temperature logs obtained by oil companies, by measurement in holes when drilling is complete, are subject to very large errors because the temperatures around the hole have been seriously perturbed by the drilling process itself; the circulation of cold drilling fluid from the surface to the bottom of the hole may change the rock temperature by some tens of degrees and it is months or years before the rock returns to its initial temperature.

Information inadequate

Energy Paper No. 9 synthesises the observational information at present available from a variety of sources and concludes that although there are some parts of the country where it is very likely that geothermal energy would be competitive with fossil fuels, the temperature information at present available is inadequate to make any overall assessment of the country's geothermal resources. Detailed cost analyses are presented to support the main recommendation that the possible benefits to the national energy budget justify the initiation of a national pro-

gramme of geothermal exploration. Although the report itself makes an excellent case for such a programme, perhaps the most telling argument is that in France today there are several district heating schemes operating competitively on low grade geothermal energy, and that a number more are planned or are under construction; these schemes have been established in situations which can be matched closely in the geology, and probably in thermal structure, by the Mesozoic sedimentary basins of this country.

It should perhaps be emphasised that although heat is continuously generated within the Earth, in most situations geothermal energy must be regarded as a depletable resource, and its exploitation as heat mining; the rate of convective removal of heat from the rocks of a hot source area is orders of magnitude faster than the heat can be replaced by conduction from below. In most cases geothermal fields are exploited at a rate designed to exhaust them in about twenty years. In a minority of situations (active volcanic regions, for example), the transport of heat to the surface by magma may match or exceed its extraction rate and the resource is not depletable, at any rate not significantly so. Questions now arise of just how and when possible geothermal resources should be exploited, and how large a contribution they might be expected to make to the national energy budget.

The answer to the first of these questions to some extent depends upon the answer to the second. If legitimate geological expectations were satisfied and suitable markets for the available energy were found, it would not be

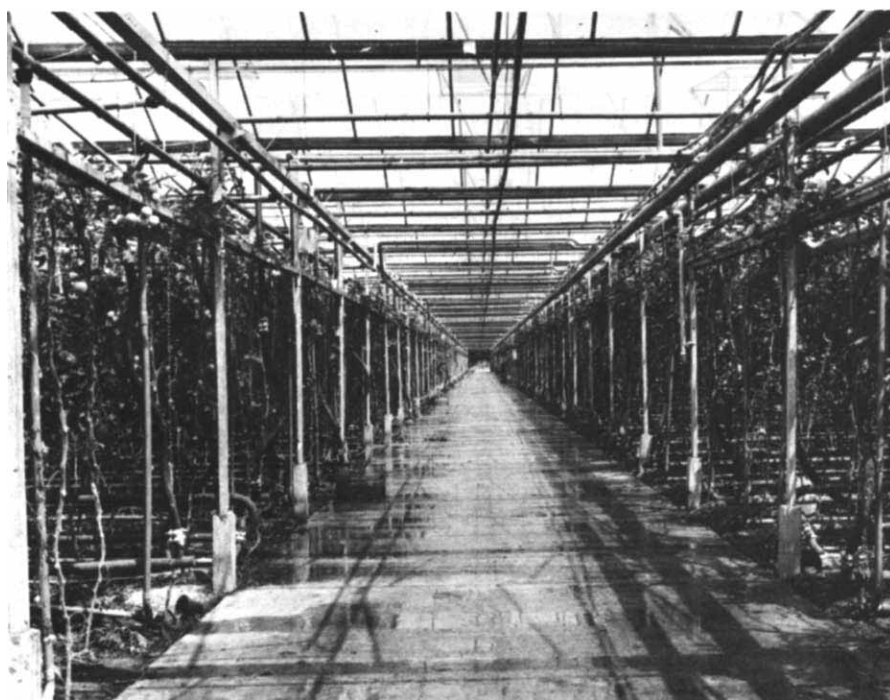
unreasonable to think of geothermal energy providing 1% or even 2% of the country's energy for 20 years. The greatest uncertainty must, however, be the availability of the market. The energy is available in the form of hot water in the temperature range 65–100 °C, temperatures which at present are generally considered too low for the efficient generation of electricity, and so the heat must be extracted from the water directly.

Furthermore, if additional heat losses by conduction are to be avoided, the hot water should ideally be used within a few miles, but certainly a few tens of miles, of the well head. There are various uses for low grade heat of this kind—domestic and industrial space heating, greenhouse heating, soil warming, fish farming and animal husbandry, various industrial fermentations, the drying of a range of organic materials and so on. It has also been shown to be economically viable to use geothermal energy for pre-heating of water, which may then be heated further by electricity, for a wider range of applications.

Policy decisions required

The problem therefore of exploiting "warm rock" geothermal resources is not purely scientific or technological. It involves either a happy geographical coincidence, by which suitable consumers who are willing to change to a geothermal supply are situated in or near a geothermal source area, or the deliberate encouragement of suitable industrial development in the geothermal areas and the use of district heating schemes in any new housing developments in the area. These clearly require major decisions of public policy. Attractive features of such developments would be the fact that warm-rock geothermal energy is virtually pollution-free—rejected warm water which has been used is re-injected into the ground for re-circulation. The surface plant is small and inconspicuous by comparison with any conventional power station.

The time scale of possible exploitation of UK geothermal resources now becomes clearer. It is reasonable to expect that the programme planned for the next three years will give reconnaissance information for much of the country. If the prospects appear reasonable at that level it would be appropriate to undertake a more intensive study of areas of particular interest. This second stage would require extensive shallow drilling (about 500 m) and some deep drilling, and could take five years; the rate of progress would be constrained by both the availability of drilling capacity and the time taken for drilling—although there is great variation, a 4 km hole could



Greenhouse heating: a use for geothermal energy

Photo: The Grower

take nearly 12 months to drill. It might then be possible to make an informed decision on whether to attempt commercial exploitation of the country's geothermal resources in the latter part of the 1980s.

If a positive decision were taken at that stage a third phase of development could begin with drilling of production holes, the planning of distribution systems and the stimulation of the potential market. The use of geothermal energy on any significant scale could not be expected before the turn of the century. This timetable is probably about the fastest which is practicable, and if desirable it could easily be lengthened; it would, however, mean that geothermal power became available about the time that production from the North Sea oil and gas fields began to drop significantly, and Britain changed from being a net exporter of oil to an importer once more.

Any consideration of the geothermal prospects of the UK, however, goes beyond the question of the heat which may be extracted from warm rocks of the upper crust. Experiments have been going on for some years at the Los Alamos Scientific Laboratory in the United States with a view to generating fractures artificially to permit water circulation both through rocks which are naturally impermeable, and through those which are so deeply buried in the crust that natural pores

and fissures have normally closed. If these experiments were successful, it could become feasible to use such "hot rocks" to heat circulating water sufficiently for the generation of electricity.

The economic viability of such a scheme would be much enhanced by improvements in deep drilling methods; at present drilling costs roughly double for every 2 km increase in hole depth. Such a development would increase the available geothermal resource by more than a factor of 10 and free it from the geographical constraints which limit the use of warm water. The national long term geothermal strategy should, therefore, be seriously re-appraised if the Los Alamos experiments are promising. But no one should pretend that geothermal energy will solve the UK's energy problem; a small and possibly highly profitable resource, on the other hand, probably does exist—it is also a resource for the exploitation of which there are no large savings of scale, so that piecemeal development to satisfy local requirements is possible.

No complacency

It is probably fair to say that in the present world economic climate a country cannot afford not to investigate its geothermal resources further. It might be asked whether it is proposed to carry out the UK's geothermal exploration sufficiently rapidly; at present there is neither the drilling capacity nor sufficient trained man-

power to mount an extensive crash programme, but even if there were it would not necessarily be more effective than a phased and progressively increasing effort over the next 10 years. The essential point is that no one be lured into a false sense of energy complacency during the next two decades of oil abundance, to the detriment of the development of other resources on which everyone will later depend.

A few may wish to ponder the point that many of the UK's large power stations reject hot water at temperatures which are regarded as suitable for geothermal exploitation in other parts of the world, and perhaps to question the wisdom of today continuing to charge a government authority with the limited mandate of generating electricity at the lowest possible price. And for those to whose lot it falls from time to time to defend the role of "pure science" in straitened economic circumstances, it is worth pointing out that the study of terrestrial heat flow which was until two years ago one of the most esoteric, albeit fascinating, branches of geophysics, has overnight become directly "relevant" and applied. Had not the Natural Environment Research Council (NERC) supported two university groups in this field for a number of years, the UK would have completely lacked the technical expertise with which to implement fully its present geothermal programme. □

Adjust, amend and heal

The face of big science is changing constantly.

Wil Lepkowski reports from Berkeley, California, on the way a famous laboratory has tried to adapt

OSCAR WILDE was once heard to say of an old acquaintance, "There goes a man with a promising past." One might be tempted to direct the same comments at the Lawrence Berkeley Laboratory of Berkeley, California, where eight men have won Nobel Prizes for work in high energy physics, nuclear physics, nuclear chemistry, and photosynthesis. All winners, save the famed, forceful Ernest Orlando Lawrence, still live and form a solid command of senior directors who continue to chart the fortunes of the facility.

It is clear that the time of grand high drama in research is over at the old Lawrence Radiation Laboratory. For one thing, its support now comes from the Energy Research and De-

velopment Administration (ERDA), whose mission is much more broad and ambitious than that of its predecessor, the Atomic Energy Commission (AEC). For another, the research budget for the things the Rad Lab always did best no longer arises at the rate it once did. And for a third, ERDA's new mission is not only much broader than the AEC's but is focusing its formula on industrial commercialisation of energy research and development, chiefly big scale systems.

Physics remains the most potent resource of the Lawrence Berkeley Laboratory (LBL). But the Laboratory is no longer the mecca for the brightest young minds seeking achievement through that once unique Rad Lab combination of men and machines. The men might still be around (and the use of the word "men" is deliberate, for the Laboratory is distinctly male

dominated), but the machines are not. High energy physics is no longer done on site. The famed Bevatron is used now for cancer research and treatment and for nuclear physics. Officials say a new and exciting era could come into being through the Positron-Electron Project with Stanford University. In that project Stanford's Linear Accelerator would inject electrons and positrons into a new storage ring and the two particles would collide to produce energy patterns of unprecedentedly fine detail. But critics note that the new machine will not be at Berkeley but at Stanford. Laboratory officials may say it doesn't matter, but it really does. The place to be will be Stanford.

Sinking sensation

One thus hears oddments of commentary in the labs, and on the bus that shuttles researchers between the University of California campus and the Laboratory, that LBL is "sinking". The precise reasons are hard to track down because the Laboratory is no longer laying people off and the research budget is rising—from the current \$47 million to around \$60 million by the end of the next fiscal year. The sinking sensation is easily

explained on psychological grounds— anxiety over a vanishing pride and autonomy. Fundamental physics is no longer choice at LBL. Money in that field—once LBL's pride—is hard to come by. "The motto at this place once was, 'why use lead when gold will do,'" comments one researcher. LBL has had to become austere since 1970 and still is searching for a guiding star.

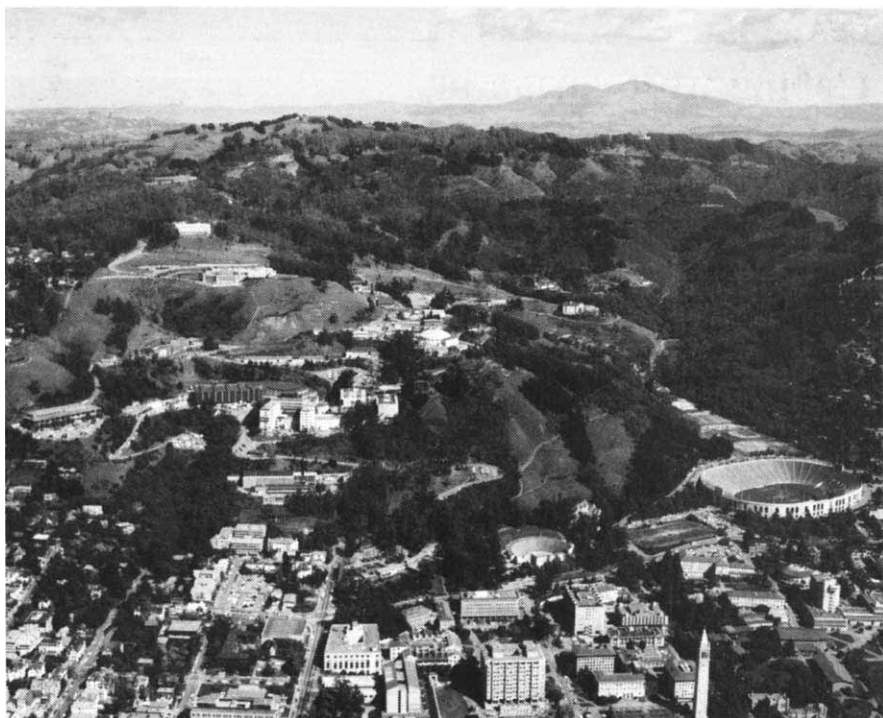
In 1970 the Atomic Energy Commission was still in existence. But its basic research budget was beginning to drop and LBL, along with AEC's seven other national laboratories, was forced to cut projects and personnel. The Laboratory formed an environmental division which with some success sought funds from agencies other than AEC. Still, the mood continued downward. As the concern over energy rose, the environment division became the energy and environment (E and E) department. By 1974, ERDA was created and with it an injection of energy research dollars. E and E will attain a budget of nearly \$17 million by next year.

E and E's acting director Robert Budnitz, a physicist, literally bubbles with optimism over the future of at least his division. Programmes include geothermal power, solar energy, fusion, and energy conservation, and the enthusiasm a visitor unerringly sees comes from scientists who like their work and know that someone who funds it appreciates it.

But LBL must still live with the fact that it is no longer the centre for intellect and excitement in basic science. ERDA, in fact, defines it as a general purpose laboratory uniquely linked to a great university. The hope is that LBL will utilise university scholars in law, economics and the social sciences to integrate energy research and technology with their application. One always wonders about the effectiveness of this kind of activity under an agency whose theme is big systems. The work may be funded but will it make a difference? And even more fundamentally, will it be any good anyway? Yet, such work is supremely important in an era of rising terrorism and nuclear proliferation.

A style that suits?

An issue that is not being tackled directly as yet is whether the management style of LBL is really suitable for the kind of work that needs doing for the development of energy research. LBL created what Alvin Weinberg has called Big Science—the need to expend large sums of money on big machines to carry out research on the smallest particles of matter. The Lawrence Laboratory owes most of its Nobel Prizes to that style of research emphasising machines, teams, and



The Lawrence Berkeley Laboratory, California

discipline. But was the work really scientifically creative? Or was it creative engineering in the service of science?

One critic, physicist Robert Yaes of Memorial University, St. John's Newfoundland, thinks LBL's science has been over-rated. He said the Berkeley group was more interested in building larger and larger machines rather than with "doing experiments with the ones they had." One of the most significant discoveries in nuclear physics, nuclear fission, he says, was made by Hahn, Strassman, Meitner, and Frisch in Europe, "even though the Lawrence group had the world's most intense neutron beam." Similarly, the first nuclear disintegrations were made by Cockcroft and Walton with an electrostatic generator that produced protons of only several hundred KeV.

"In short", he says after citing other examples, "even though the Berkeley group possessed beams of energy and intensity available nowhere else in the world, the most significant discoveries were still being made by the 'string and sealing wax' people in Europe".

There is growing debate over whether large scale technology is in society's best interests, with its tendency toward more and more control by a technical elite, towards the necessity of producing elaborate security structures around it, and toward increasing social fragility out of the possibility of breakdown. It may be that at least one highly sophisticated laboratory would serve ERDA well by emphasising the "string and sealing wax" approach to energy research and development through a

focus on small systems. At the very least ERDA might put aside one laboratory to question prevailing philosophy and probe the basics of energy systems. At the moment it has no such place—a place that preserves and nourishes first principles.

ERDA taking a view

ERDA is studying the future role of the national laboratories in light of its mission to commercialise energy technology. It is asking practical questions about how one goes about linking their research and development functions with industrial development and demonstration. In the case of LBL, it is serious about integrating research at the University of California, which is contract manager of the Laboratory, with research at LBL.

But ERDA is also undecided about its overall research management strategy. It talks of regional programmes and projects and speaks of using the national laboratories somehow in the regionalisation of energy research. At the same time it says many laboratories, especially Berkeley, have a broad spectrum of capability in many fields. And all the while, ERDA policies are subject to political whims, since their Associate directors are all politically appointed. It is little wonder LBL officials are confused and worried. It is a centre with a rich tradition in research, and once bursting with pride and confidence. Now it searches for a prevailing philosophy against a backdrop of an agency that cannot yet say exactly how it plans to steer its own ship. □

USA

Coping with variability

Wil Lepkowski reports on the response of US agriculture to climatic change, the subject of a recent report

THE one sure thing about the weather—and the climate—is that it will change. Therefore farmers, who depend on the weather, must be nimble. That is the essence of a report issued last month by the Washington-based Institute of Ecology under support from the Kettering Foundation of Dayton, Ohio. The report says climatologists may not agree for some time over whether the climate is warming or cooling. But what they can agree on is that it will fluctuate. The strange point the report makes is that North American agriculture may be unprepared for the normal fluctuations in climate.

Why? It may have grown complacent over the good run of weather experienced during the 1950s and 1960s, when agriculture boomed. As a result, the technology of fertilisation, pest control, and plant genetics may have softened crop resistance to fluctuation with the result that agriculture may have made itself vulnerable to trouble.

The panel, which had only six months to study the issue, makes three

major recommendations: that weather scientists step up research on the processes and mechanisms of climatic change, that scientists develop research leading to improved strategies for food production under climatic stress, and that governments expand agricultural information networks to inform farmers more quickly of conditions affecting their crops.

The saga of the American farmer is that of a crusty realist always prepared for climatological vagaries. But this report emphasises agriculture's soft underbelly—that behind the legend lies bureaucratic complacency. It says with world population continuing to expand by 200,000 souls a day, and virtually all arable land under cultivation, agriculture is at the brink. It must be more watchful of its assumptions, more anticipatory of trouble. And the bureaucracy isn't.

The study, which was chaired by Professor James E. Newman, a Purdue University bioclimatologist, is ecology's first major try at tackling the agricultural policy issue. Its range was limited to Canadian wheat and American wheat, sorghum, corn, and soybeans, and it traced the production record of these crops under various degrees of climatological stress during the past 40 years. Its tone is that of a

gentle warning rather than a vigorous call to action. One senses that farmers gathered for gossip at the general store would nod tired agreement but that the boardrooms of agribusiness corporations would pay only passing heed.

There is an undercurrent of concern over the vulnerability of technological agriculture, especially in view of its dependence on fossil fuel technology. And climate remains a concern. Four big studies are continuing in the US and each bears watching:

- "Study of Changing Climate Patterns and their Effect on Agricultural and Renewable Resource Productivity", by the National Academy of Sciences.

- "Living with Climatic Change", co-sponsored by the American Meteorological Society, the Canadian Meteorological Society, the Geophysical Institute of the National Autonomous University of Mexico, and the Science Council of Canada.

- The Large Crop Inventory experiment, by the National Aeronautics and Space Administration, US Department of Agriculture, and National Oceanographic and Atmospheric Administration.

- "Impact of Climate Change on the Character and Quality of Human Life", by the Aspen Institute for Humanistic Studies. □

BRITAIN

Genetic manipulation: report due

For many of Britain's molecular biologists, a long wait is almost over. Eleanor Lawrence reports

THE long-awaited report of the Williams Working Party on the Practice of Genetic Manipulation is "being printed and will be published shortly", according to Mr Fred Mulley, the UK Secretary of State for Education and Science, in a written reply to a Parliamentary question last week.

Mr Mulley was giving few details away on the code of practice itself, except that it will in the first instance be a voluntary one, with the possible introduction of specific statutory controls later in the light of experience. It will set out principles for categorising experiments and recommend various levels of safety precautions necessary for particular categories of experiment. But he shed a little light on the intended role of the proposed central advisory group. From this it looks as though scientists will have to wait until the advisory group is set up

before their long-deferred experiments can begin.

The provision of a central advisory service for laboratories wishing to carry out genetic engineering was originally recommended by the Ashby Working Party which reported in January 1975. The experiments involve the transfer of alien genes to the common gut bacterium *Escherichia coli* by splicing the foreign genes onto a plasmid or bacteriophage vector.

One consequence of this might be that *Escherichia coli* with unusual and possibly harmful properties conferred on it by the transplanted genes might escape from the laboratory and infect the population at large. One of the fears when these experiments were first proposed was that they would be carried out in laboratories with little previous experience of the microbiological techniques developed to contain potentially dangerous organisms.

The Williams Working Party has endorsed the idea of a central advisory service but apparently sees it as a watchdog as well as adviser. According

to Mr Mulley's statement the Working Party has recommended that any laboratory contemplating experiments likely to come under the code should be required to notify the central advisory service before beginning work. This recommendation can be implemented through regulations which can be drawn up under the present Health and Safety at Work Act.

These recommendations will in some respects provide the proposed British scheme with an advantage over the recently-published American guidelines, which only strictly apply to work funded by the National Institutes of Health; in Britain, all laboratories, whether university, government or industrial, will be covered.

The Working Party report is expected within the month, and Mr. Mulley intends to appoint the members of the central advisory group as quickly as possible and make immediate arrangements for it to get to work. The Health and Safety Commission will also soon be circulating for comment its proposals for regulations requiring notification of proposed experiments. □

UK ENERGY

● The UK Energy Secretary, Mr Anthony Wedgwood Benn, rarely out of the news these days, has been asked to delay his autumn decision on the 1,300 MW demonstration fast breeder reactor, CFR-1, pending the forthcoming report from the House of Commons Select Committee on Science and Technology on alternative energy sources. The request came last week from Mr Arthur Palmer, the Committee's Chairman, and in response Mr Benn has indicated that he does not at the moment want to wait beyond the end of October.

This tight autumn schedule is not new, but Mr Benn's implication that he may not wait is. What is more, the UK Atomic Energy Authority (UKAEA) this time round is believed to be seeking clearance only for a firm timetable and site selection, and is prepared to wait as long as two years for a final decision on whether to build the fast breeder. As for the Select Committee, which is considering the likely future role of the reactor in Britain for the purposes of its report, it is understood to be more disposed towards the reactor than against it at present.

The UKAEA's drive to keep progress on the fast breeder ticking over smoothly is meanwhile slipping into a higher gear following Treasury approval of a £10.5 millions contract for three steam generators. The generators, expected to be ready for the 250 MW prototype fast breeder at Dounreay in Scotland by 1979, are exactly similar in design and size to those planned, in greater number, for CFR-1. The design in use at the moment is unlikely to prove entirely satisfactory for commercial operation, and represents little more than an interim stage of development.

Also making progress, it seems, is the Central Electricity Generating Board's (CEGB) attempt to negotiate a share in the 1,200MW Superphénix project on the Rhône. Discussions have been proceeding for some months, but details have remained secret. Now it is reported that the French-German-Italian consortium involved has offered the CEGB a 10% interest for an initial stake of £60 millions.

● Why has Mr Benn appointed Mr Francis Tombs, head of the South of Scotland Electricity Board, as successor to Sir Peter Menzies, Chairman of the UK Electricity Council, when he retires in March 1977? Insofar as it is a matter of alternatives, the simple answer is that Tombs is only 52, whereas his counterpart at the

CEGB and his obvious competitor, Sir Arthur Hawkins, is himself close to retirement at 64.

But, like most problems facing the Energy Minister, this is not being seen as a simple matter of alternatives. In his present position, for instance, Tombs heads the only electricity board in the country which both generates and markets electricity. So, one argument runs, the government could be preparing to implement the Plowden Report's call for closer integration of both aspects of the business throughout Britain.

More controversially, there is the suggestion that Tombs' support for the Steam Generating Heavy Water Reactor (SGHWR), now under review by the Department of Energy (DEN), won him his new position—that he would be able to keep the SGHWR programme firmly on course. Equally, though, with Tombs placed where he may have to compromise with individual generating boards, the project might more easily slip quietly away.

Not so, says the Department of Energy, chary of every hypothesis. Then why has Tombs been appointed so far in advance? Once bitten, twice shy, says the DEN, adding that it burned its fingers over the delay in the recent reappointment of Sir Derek Ezra as head of the UK National Coal Board.

● The SGHWR receives a mention in the report released last week of the Public Accounts Committee (PAC), the committee of MPs which oversees government spending. In the interests of "effective financial and technical control", it says, the financial responsibilities of the various parties involved "should be clearly defined as soon as possible". Every inducement to efficiency and economy should be incorporated in the contractual arrangements, and the absence of a fully costed design should not delay agreement in principle on these matters. The committee was told that the total cost of the SGHWR programme would be about £100 million.

The committee also made some comments on British Nuclear Fuels Ltd (BNFL). BNFL had in the previous week published its annual report showing an increase in both profits and export earnings; Alex Eadie, junior minister at the Department of Energy, had at the same time revealed in a Commons answer that the government had agreed a massive £715 million investment programme for the next ten years.

BNFL (profits last year: £11.9

million) has paid no dividends for the past four years; this, says the PAC, is equivalent to the Exchequer providing interest-free advances of capital, and the no-dividend policy should be re-examined if the company is to operate on a normal commercial basis. There should also be close control of BNFL's investment programme; expenditure should be approved only if there are good prospects of an adequate return.

The main feature of BNFL's investment programme is the expenditure of £300 million enlarging the capacity of its uranium enrichment facility at Capenhurst in Cheshire. The plant, based on the gas centrifuge process, involves BNFL working in association with West Germany and Holland as partners in Urenco, which last year won sizeable enrichment orders. Another £130 million focuses chiefly on the conversion of uranium oxide to uranium hexafluoride for enrichment, for which BNFL also won orders last year; £245 million is going on extensions and improvements to the facilities at Windscale used for reprocessing nuclear fuel; and £40 million is being devoted to developing the process for the vitrification of waste.

According to Mr Eadie, the saving to the balance of payments through Magnox fuel reprocessing for domestic customers is "at least £200 million", and the revenue from reprocessing carried out at Windscale for overseas customers has so far been "about £12 million".

● A British Government reply made recently to the 42 recommendations on energy conservation from the Select Committee on Science and Technology last year latches on to some of the committee's ideas but parries the criticisms about weakness in central direction on conservation by saying that there are "limits on how active a part government can play without getting into a situation where it is taking consumers' decisions for them". That said, the government has gone some of the way to meeting the committee's call for a full-time task force of Ministers and others, and has also announced more help for small companies, an extension of the energy-saving loan scheme for companies, the provision of better information on energy-saving, and an extra £1 million for the "Save It" campaign (described by the Select Committee as a "feeble" exercise). If these moves are more than marginally effective they will need to be factored in to the ongoing debate on Britain's future requirements of nuclear power.

IN BRIEF

More nuclear deals

There's no relief in sight for those campaigning for greater restraint in the burgeoning international trade in nuclear technology. At the end of last week the delayed Franco-South African deal for the construction of two pressurised water reactors finally went through when Escom and the French consortium headed by Framatome signed contracts in South Africa. Only a couple of days earlier France's Foreign Trade Minister initialled an agreement in Seoul under which Framatome will build two similar reactors for South Korea.

France's sale of a small reprocessing plant to South Korea was stalled earlier this year following US pressure. Now similar pressure has been brought to bear over an almost identical deal between France and Pakistan. At the weekend Dr Henry Kissinger told

President Bhutto that Pakistan risked losing US military and economic aid if it went ahead. Bhutto reportedly insisted that Pakistan be treated in the same way as Iran, which apparently wishes to reprocess nuclear waste from its reactors locally rather than outside the area. Israel and Egypt, on the other hand, with which the US has now initialled agreements on the sale of reactors, have agreed to let any reprocessing be done outside the Middle East.

In the US, meanwhile, the State Department has conceded that American materials may have been involved in India's nuclear explosion in 1974.

Biblis problems

A senior executive of Kraftwerk Union (KWU) announced this week that the huge nuclear power plant at Biblis, West Germany, would be technically

capable of returning to service by the end of the week, although clearance from the relevant authorities would still take some time.

The twin pressurised water reactors were closed for a routine inspection three months ago. In a joint statement last week, the operators, RWE, and builders, KWU, disclosed that "incipient cracking" had been found in the cooling water reservoirs of both reactors, and that steel bolts which had sheared off in the main cooling circuit of the commercially operating 'A' block reactor had been carried close to the nuclear core. The shutdown was originally scheduled to last eight weeks. The Biblis installation is the model for the four reactors to be built by KWU in Brazil and Iran, and officials are stressing that the breakdowns are not a cause for alarm. One estimate which has been made of the cost to the operators is \$50 million.

JULY is the prime time for the High Country in the southern Sierra Nevada of California. This was so in 1976 more than usual, because of the severe drought that had prematurely melted the big snowbanks. It was in July 1935 that I had first tasted their icy waters, as addictive as the most insidious of narcotics. And so, for the 39th summer, in a series broken only by war years, I set forth to regions above timberline, knapsack on back. What new changes would I find that had been wrought by technology and human beings?

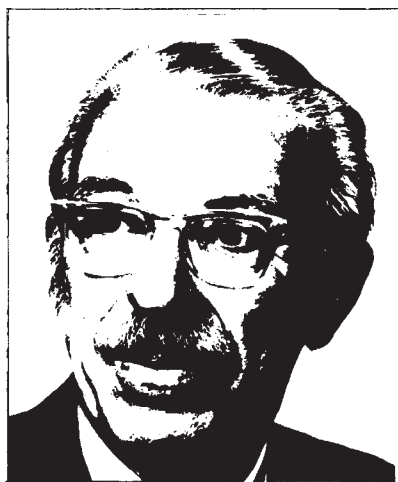
Within the mountains of California, Yosemite, Kings Canyon and Sequoia National Parks are known for small areas that can be reached by road, but beyond these are hundreds of square miles that are reserved for what has been called "the aristocracy of the physically fit." Even this elite group dwindles as one heads away from the trails. It is curious that, even in the mountains, human beings tend to collect in herds. I still have no difficulty in finding big lakes, above 11,000 feet, where I am alone with peaks, trout and jet planes.

Thirty years ago, many beauty spots of the High Country were defiled by monumental piles of cans and bottles. Most of these have been patiently removed by volunteer labour. The volunteers, helped by officials, have also removed wrecked planes, piece by piece. Travel by horses and mules has been greatly reduced by high costs, and has been mostly replaced by the use of ingen-

ious lightweight backpacking equipment and dried food.

The US Government, as may be expected, increasingly regulates travel in the wilderness. The official and praiseworthy concept is that "man as a temporary visitor, should leave no permanent imprint." One has to

Mountain streets



THOMAS H. JUKES

file the equivalent of a "flight plan" before being permitted to take off. Simultaneously, nine pages of "suggestions" and rules are thrust into one's hand. Enforcement of these is often by female rangers who are the spiritual granddaughters of members of the Women's Christian Temperance Union. One of these rangers is spoken of with admiration by her col-

leagues as having recently written twenty "traffic tickets" for offences committed by feckless hikers on the eastern slopes of Mount Whitney.

Years ago, the "organic waste problem" in mountain travel was officially given only a casual reference. We were archly advised to "do like the cat; dig a hole and bury it." Today the instructions for "sanitation and waste disposal" occupy seven column inches. The dimensions of the pertinent hole are now specified as 5 to 6 inches deep and 8 to 10 inches across. I notice that the indigenous cats (*Felis cougar*) apparently do not read, and do not dig holes, preferring to leave evidence of their passage in the open as a defiant challenge to territorial intruders.

On July 15, I lay for twelve hours in a small plastic tube tent, at 10,800 feet, as a magnificent electric storm crashed incessantly in the darkness and, at midnight, a huge rain-loosened rock avalanche roared down a nearby granite cliff. Next day, in the bright sunlight, I caught golden trout. A few nights later, a Pacific Fisher (*Martes pennanti*) stealthily and silently made off with a string of golden trout that I had hung above my head in a pine tree while I slept in the open, well above the altitudes where bears live. My vexation was tinged with admiration when I got up at dawn to find the loss. I headed for the lake to replace the theft, and then walked for nineteen miles, through the rocky streets of the mountains, back to the road-head.

news and views

Chromatin superstructure

from H. G. Davies

PROGRESS in understanding the structure of chromatin and eukaryotic chromosomes continues apace. Early electron micrographs by Ris and others had shown that the long fibres obtained from chromosomes were either thin, about 10 nm, or thick, about 25 nm, depending on the presence or absence of chelating agents. Hitherto, however, it has not been clear how the two fibres are related. The past few years have seen the emergence of evidence that chromatin has a subunit structure; nuclease digestion of chromatin yields pieces of DNA which are multiples of about 200 base pairs, histones occur as oligomers, and electron micrographs of suitably lysed nuclei show linear arrays of interconnected roughly spherical particles about 10 nm diameter called beads, ν -bodies, or nucleosomes. Kornberg has put forward a model for the 10 nm fibre—a flexible chain of repeating structural units, like “beads on a string” (*Science*, **184**, 868; 1974), each bead containing two each of the four major histones.

Finch and Klug (*Proc. natn. Acad. Sci. U.S.A.*, **73**, 1897; 1976) now provide evidence to show that thin fibres about 40 repeat-units long, obtained from rat liver nuclei by brief nuclease digestion, can undergo helical folding to form a solenoidal structure about 30 to 50 nm wide. The evidence for helices is based first on electron micrographs which show more-or-less clear striations, separation about 12–15 nm crossing the thick fibre, and second, on new X-ray diffraction data and a re-interpretation of the earlier patterns obtained by Wilkins and collaborators, and Luzzati and Nicolaieff, showing maxima 11.0, 5.5, 3.7, 2.7 and 2.2 nm. The key experiment is a comparison of the X-ray spectra of thin and thick fibres and the results are different from those obtained previously, due, presumably, to improved methods of preparing chromatin. Sperling and Tardieu (*FEBS Lett.*, **64**, 89; 1976) find that the scattering from thin fibres in the region of spacings 40 to 3 nm fits the theoretical curve for a continuous density rod with no peaks at 11 and 5.5 nm. The 11 nm and other reflections appear, however, under conditions where solenoidal structures can be seen in electron micrographs. Hence Finch and

Klug now propose that the reflections at about 11 nm and higher orders arise from the spacings between the turns of solenoids of low pitch angle and not from an interbead spacing of 10–11 nm along the thin fibre as had been universally, and quite reasonably, assumed, and indeed used by Kornberg to support his model. The new explanation of the spectra is similar to that originally proposed by Pardon and Wilkins (*J. molec. Biol.*, **68**, 115; 1972) except that they thought in terms of supercoiling DNA, rather than the continuous 10 nm fibre. The data appear to confirm a suspicion held by many that the repeating unit in the thin fibre only becomes visible as ν -bodies or nucleosomes under special conditions. Those electron microscopists who, for one reason or another, did not see the thin fibres as beaded, but continuous, a result confirmed by Finch and Klug, will be unable to resist a smile at this latest turn of events.

Another key observation is that by Carpenter *et al.* (*Nucleic Acids Res.*, **3**, 1739; 1976) on neutron diffraction from oriented fibres of chromatin. The meridionally oriented reflection at about 10 nm, is now seen to be split, forming a cross pattern with semi-meridional angle of 8° to 9°. This is a sure sign to the cognoscenti that it originates from a helix. They propose as a model a coil of nucleosomes of pitch 10 nm and an outer diameter about 30 nm, dimensions corresponding to about six nucleosomes per turn of the helix. Of course, such a splitting of this meridional reflection could also be expected in the solenoidal model formed from a continuous 10 nm thread.

On the basis of electron microscopy of sections, we have described thread-like units within condensed interphase chromosomes, or chromatin bodies. These “superunit threads” about 28.0 nm diameter, are clearly recognisable because they tend to line up into layers at the surfaces of nuclei, and can also form monolayers, width independent of species, plant and animal. Davies and Haynes (*J. Cell Sci.*, **21**, 315; 1976) now report that nucleoli are attached to the nuclear envelope by a monolayer of these superunits. We have suggested

that the superunit thread is formed from some type of helical arrangement of a subunit thread—“close-packed beads-on-a-string”. This would account for the earlier observation that the unit appears tubular. Finch and Klug comment that their solenoidal model being of similar geometry can be equated with superunit threads found within nuclei.

Much clearly remains to be learned about the detailed molecular structure of these proposed helically arranged repeating units, how they are further folded up in mitotic chromosomes and how they unfold in those parts of interphase nuclei where RNA transcription occurs. Also the way the DNA molecule is folded around the repeating unit is not clear, but results on sections suggest the repeating unit is asymmetric with more DNA on an inward pointing face. The spiralling of mitotic chromosomes and the helical nature of the DNA molecule have long been known and the above results will come as no surprise to those who believed that there were orders of coiling in between. The point is that speculation is now being replaced by fact. □



A hundred years ago

In the *Bulletin International* of August 3, M. de Tastes relates some interesting particulars of a waterspout (*trombe*) which was observed near Tours, on May 25 1876. It first appeared as a mass of whitish vapour against a background of dark-coloured clouds, which gradually assumed the form of an inverted cone pointing to the ground, and terminating in a long sinuous band. A whitish sinuous column soon appeared suddenly between it and the ground, and rapidly enlarged upwards, the whole phenomenon soon assuming the appearance of two cones united at their summits. The lower cone, at first lightish-coloured and in a certain degree transparent, gradually assumed a darker shade, which was propagated from the base towards the summit.

from *Nature*, **14**, August 10, 320, 321; 1876.

Anaesthetic mechanisms

from C. D. Richards

ANAESTHETICS affect every variety of living system, from microorganisms to mammals; they are very nonspecific drugs which depress a whole battery of cell functions including cell motility, cell division, photosynthesis, oxidative metabolism and electrical excitability. Their mode of action poses one of the fundamental problems in biology. Their clinical value rests solely on their ability (at low concentrations) to impair certain higher nervous functions such as consciousness and memory without disrupting the function of the heart and respiratory system.

Four key observations suggest that anaesthesia is the result of some physical interaction of the anaesthetic with certain constituents of the cell rather than a specific chemical reaction. First, the effects of anaesthetics are freely and rapidly reversible; second, the potency of an anaesthetic is directly related to its lipid solubility; third, anaesthetic properties are shown by a wide variety of chemically unrelated substances; and fourth, the effects of anaesthetics can be reversed by high pressures.

The critical volume hypothesis first discussed by Mullins more than 20 years ago (*Chem. Rev.*, **54**, 289; 1954) seems to be the most successful of the various hypotheses proposed to explain the action of anaesthetics. In its simplest form it proposes that anaesthesia (narcosis) occurs whenever a critical fraction of anaesthetic has been achieved in the membranes of the cell; this critical volume is held to be dependent on the species under study but independent of the nature of the anaesthetic.

In its original form the critical volume hypothesis added little to the well established Meyer-Overton concept of a role for lipid solubility in the mechanism of anaesthetic action, but recent investigations of the reversal of anaesthesia by pressure by Miller *et al.* (*Nature*, **231**, 368; 1971; *Molec. Pharmac.*, **9**, 131; 1973) have led to a more satisfying model. They propose that anaesthetic dissolves in some hydrophobic region of the cell—presumably lipid—causing that region to expand, thereby impairing some vital function. From the amount of pressure required to reverse the effects of various doses of anaesthetic they calculated that during general anaesthesia the hydrophobic region should expand by about 0.4% (v/v). This prediction has subsequently been verified by Seeman and Roth (*Biochim. biophys. Acta*,

255, 171; 1972) for erythrocyte membranes.

This still leaves four important questions. How is the membrane expansion brought about? How does the membrane expansion lead to impaired membrane function? Which of the many functions of the membrane are impaired by anaesthetics? Does anaesthesia result from the impairment of one or several specific functions?

Membrane effects

Metcalf *et al.* (*Mol. Pharmac.*, **4**, 87; 1968) found that benzyl alcohol which can act as an anaesthetic increased the fluidity of biological membranes. This observation was later extended by Trudell *et al.* (*Biochim. biophys. Acta*, **291**, 328; 1973) to include clinically important anaesthetics, such as halothane. It was subsequently proposed that anaesthetics cause membrane expansion by increasing the disorder of the fatty acid chains of the phospholipids of the membrane bilayer. During general anaesthesia, anaesthetics occupy only 0.2% (v/v) of the membrane whereas membrane expansion is of the order of 0.4%. Seeman has shown, however, that artificial phospholipid bilayers only expand by 0.2% and so he proposed that changes in the conformation of the membrane proteins are the cause of the large expansion of natural membranes (*Experientia*, **30**, 759; 1974). Furthermore, in a recent paper Boggs *et al.* (*Molec. Pharmac.*, **12**, 127; 1976) have shown that concentrations of anaesthetic sufficient to cause general anaesthesia or which block the conduction of impulses along a nerve ("local anaesthesia") have little or no effect on the fluidity of artificial membranes. Although such observations cast doubt on the view that anaesthetics impair membrane function through a generalised perturbation of the structure of the lipid membrane they are consistent with the view that the interface between lipid and protein may be the site of anaesthetic action.

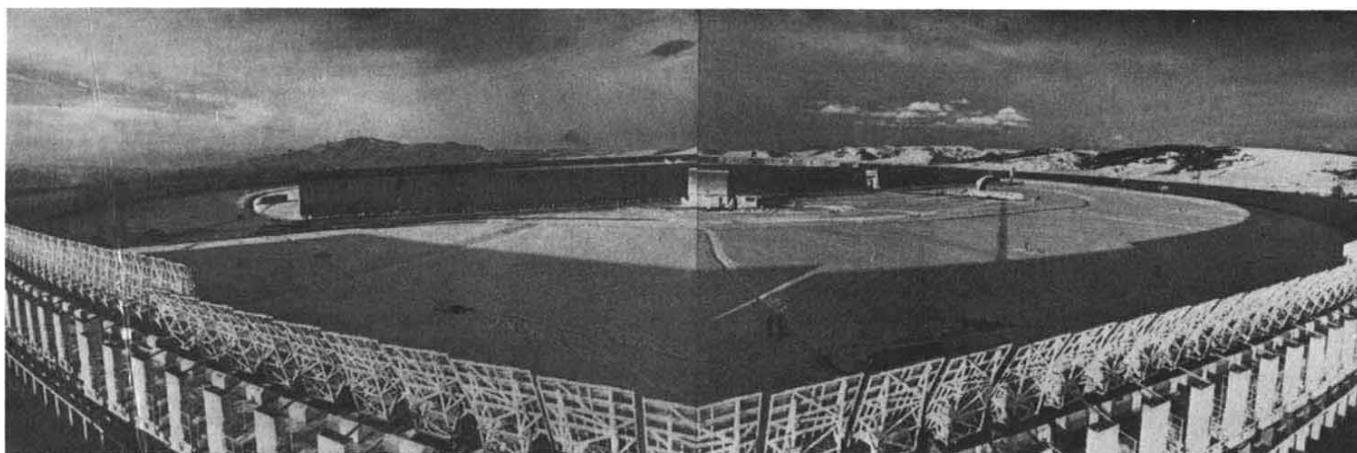
There is now evidence to suggest that at least some membrane-bound proteins require the presence of a layer of tightly bound lipid to stabilise them in their active form. This has been shown for cytochrome oxidase (Jost *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **70**, 480; 1973) and for the calcium transport protein of the sarcoplasmic reticulum (Warren *et al.*, *Nature*, **255**, 684; 1975). By studying the effects of temperature

on the calcium transport protein reconstituted in defined lipid mixtures, Warren *et al.* (*Biochemistry*, **13**, 5501; 1974) showed that the enzymatic activity of the complex decreased as the lipid became more crystalline. This suggests that the activity of this protein is governed by the fluidity of the lipid in which it is embedded. In the light of this data, Lee (page 545, this issue of *Nature*) has proposed that the sodium channel in nerve membranes may be surrounded by a ring of rigid lipid molecules that serves to keep it open. He suggests that anaesthetics block nerve impulse conduction by fluidising this boundary lipid so causing the sodium channel to collapse inwards. One consequence of the model is that a nerve blocked by an anaesthetic should have its conduction restored when the temperature is lowered, and this has indeed been shown for the action of ethanol on squid axon (Spyropoulos, *J. gen. Physiol.*, **40**, 849; 1957).

Specific interactions

It is not difficult to envisage other membrane proteins such as receptors being similarly surrounded by boundary lipid which serves to keep the protein poised in an optimal conformation. The fact that some anaesthetics affect one function rather than another could then be explained by relatively specific interactions either with the boundary lipid or with the individual protein. Certain volatile anaesthetics have been shown to inhibit the bacterial enzyme luciferase apparently by competing for the substrate binding site (White, in *Molecular Mechanisms in General Anaesthesia*, Ch. 13, Churchill-Livingstone, London, 1974; Middleton and Smith, *Proc. R. Soc. Lond.*, **B193**, 173; 1976).

Moreover, specific interactions of this sort could account for the antagonism of the depressant effects of the steroid anaesthetic alphaxalone by the non-anaesthetic steroid $\Delta 16$ alphaxalone observed in isolated preparations of mammalian brain tissue (Richards and Hesketh, *Nature*, **256**, 179; 1975). Nonetheless, such ideas must remain speculative until it becomes possible to isolate receptors and reconstitute them in artificial membranes of defined composition. With the recent developments in understanding the role of lipid-protein interactions in membrane biochemistry, the next period of anaesthetic research should be an exciting one. □



New giant Soviet radiotelescope

from Boris Belitzky

THE RATAN-600 radiotelescope, under construction at Zelenchuk in the North Caucasus since 1968, is now completed and fully operational. RATAN-600 is an acronym derived from the Russian for "radio-astronomical telescope of the Academy of Sciences" and a figure approximating the 576-m diameter of the telescope. Zelenchuk is also the home of the world's biggest optical telescope, the fully-operational 240-inch BTA reflector. Together with a large neutrino detector, under construction nearby, this unique array of instruments comes under the general umbrella of the Special Astrophysics Observatory founded there by the Soviet Academy.

Unlike Britain's Mark I instrument at Jodrell Bank or the slightly bigger West German instrument in the Effelsberg mountains, RATAN is not a steerable dish, but embodies the variable profile concept.

Despite its size—its geometric area equals 10,000 square metres—the new radio telescope has been designed to operate in the short-wave spectrum down to 8 mm. (Cornell University's Arecibo instrument, by way of comparison, stops short at 10 cm.). As for RATAN's upper limit, this was selected to take in both the 18-cm hydroxyl wavelength and the 21-cm neutral hydrogen wavelength.

It is at the less well explored, shorter end of this spectrum, however, that the designers (a Pulkovo team under Dr Y. N. Pariisky and Dr N. L. Kaidanovsky, with support from Moscow University) expect it to yield the most valuable results, and RATAN's record sensitivity and resolving power are, accordingly, in that region. Its aerial comprises a circular reflector, a flat reflector, and several feeds. The circular reflector is a variable profile aerial consisting of 895 cells mounted on a circular foundation and capable of tilt-

ing, rotating and moving radially. The flat reflector, mounted in the southern part of the circle, consists of 124 cells that can only be tilted.

The principal mode of operation requires pointing the telescope's axis at a source and observing its passage through the aerial pattern. Observations of sources near the horizon involve approximately a quarter of all the reflecting cells and can be conducted according to four independent programmes simultaneously. At angles of 30 to 80° about a third of the cells are operational and three independent programmes can be pursued simultaneously. Finally, zenithal observations involve the entire circular reflector, and the telescope has a pencil-beam pattern equivalent to that of a continuous-surface reflector 576 m in diameter.

Each cell is fitted with pickups, which control all its movements and adjust it to the exact position required. Feed movement takes place along 12 radial tracks spaced azimuthally at 30° intervals. A turntable in the centre of the circular reflector serves to shunt a feed from one radial track to another. Apart from the variable profile mode, the flat reflector makes it possible to use the telescope as a parabolic cylinder according to the Kraus system. In this case the telescope has a constant knife-edge pattern, and the time needed for sky surveys is substantially reduced. By combining the circular and flat reflectors in the southern sector it is possible to use the telescope in certain other operational modes, such as tracking a source. This mode of operation is, of course, a particular asset wherever maximum sensitivity is required.

RATAN was begun in 1968, and the first part of the project—the northern sector of the circular reflector—became operational in 1974. Some significant

astrophysical results were obtained during the running-in period. These included the discovery of a solar "radio granulation"—a fine structure of the Sun's radio emission in the centimetre range, which appears to coincide with its chromospheric net. The radio astronomers at Zelenchuk have been able to observe the radio emission of the solar corona at large angular distances from the Sun and to observe quasars through the corona.

Simultaneous observations of radio sources on several frequencies have yielded their instantaneous spectra and permitted the sources to be separated into components. One of the most interesting findings concerning the structure of radio sources has been the discovery of a bar linking the components of such well-known double sources as Cygnus A. There have also been measurements of the Einstein effect—beam deflection in the Sun's gravitational field.

Now that the telescope is fully operational, the programme of research is being expanded. Inside the Solar System it is proposed to study physical conditions on the surfaces of the planets, their satellites, and the minor planets. Solar studies, which command growing interest among Soviet astronomers, will include investigations of the brightness distribution in the active regions of the Sun to determine the structure of magnetic fields and condensations in the lower corona and the upper chromosphere, and also the structure of the chromosphere and the transition zone.

Further afield there are to be radio observations of the Galaxy and of the Solar System neighbourhood, of the Galactic nucleus, of the fine structure and kinematics of gaseous and planetary nebulae, and of pulsars and the nearest stars.

Extragalactic studies, which in Pariisky's view are the most interesting application for RATAN, will be conducted mainly in the decimetre range. Naturally, there is particular interest in studying quasars and radio galaxies

with anomalously intense radio emissions in the hope of learning more about the nature of these objects and the sources of their energy. There are also to be studies of the fine structure of many radio galaxies, with particular reference to their brightness distribution spectrum and magnetic field structure. According to Dr Lev Gindilis, who has been concerned with planning the RATAN sky surveying activities, these are to be conducted on a systematic basis and at the highest frequencies possible. This is prompted by the paucity of information in this range and the hope of observing quasars at the initial stage of their evolution. Gindilis considers RATAN to be an optimal instrument for surveys in the entire range from 4 mm to 20 cm, in which there is a minimum of natural interference for observing cosmic sources. RATAN sky surveying work will also take in account the wishes of radio astronomers interested in the problem of communication with extraterrestrial intelligence (CETI).

Ultimately the RATAN control complex is to be linked to the main radio-astronomical centres of the Soviet Union, with the result that the potential of this giant instrument will be used more effectively. RATAN-600 is also seen by Soviet radio telescope designers as a stepping stone towards far more powerful facilities. □

Aspects of plasmid behaviour

from J. R. Saunders

A NATO Advanced Study Institute on the Biology of Bacterial Plasmids was held at Kavouri, Nr Athens from July 5-16, 1976.

INTEREST in the biology of bacterial plasmids has been stimulated both by their use as cloning vehicles for genetic engineering and by the increasing knowledge that a multitude of important bacterial properties are plasmid specified. The role of R plasmids in mediating antibiotic resistance in clinically important bacteria is well known. Bacterial pathogenicity for plants can, however, also be encoded by plasmids. N. Van Larebeke (University of Ghent) showed that the ability of *Agrobacterium tumefaciens* to cause "crown gall" tumours in wounded plant tissues is determined by tumour-inducing (Ti) plasmids. Furthermore tumorigenic ability can be transmitted to other *Agrobacteria* either by transformation or by conjugation mediated by the R plasmid RP4. M. Holsters from the same group demonstrated that Ti plasmids allow

host bacteria to synthesise and utilise either nopaline or octopine. It is interesting that tumours produced by *A. tumefaciens* contain, (depending on the strain) either of these unusual amino acids, suggesting that certain plasmid genes may be expressed in tumour cells.

P. Williams (University of Wales, Bangor) hailed the 'degradative' plasmids of *Pseudomonas*, unlike most plasmids, as being totally beneficial to the biosphere as a whole. The ability of some *Pseudomonas* species to degrade and utilise many natural and synthetic organic substrates is due in part to the existence of plasmids determining the enzymes of specific catabolic pathways. The manipulation of these plasmids could therefore be used to construct bacterial strains capable of 'mopping-up' oil spillages and industrial wastes.

The translocation of resistance genes continues to generate interest. S. N. Cohen (Stanford University) stated that Transposon A (TnA) encoding resistance to ampicillin comprises part of the larger transposon TnS which specifies resistance to ampicillin, streptomycin and sulphonamides and that TnA may provide the driving force for the translocation of the larger unit. Evidence for interactions between transposons was also presented by M. H. Richmond (University of Bristol). He reported that the presence of TnA on a plasmid reduces the frequency of transposition of a second copy of TnA to it by a factor of 10,000 in recombination-deficient (*rec*⁻) cells. On the other hand, in *rec*⁺ cells the presence of a resident copy of TnA merely reduces the frequency of accretion of a second copy by 90%. In the latter case however the incoming copy of TnA apparently replaces the resident TnA probably by reciprocal recombination rather than transposition. The results therefore suggest that one transposon in some way interferes with the *rec*-independent translocation of like elements. The as yet unpublished work of several laboratories on the genetic structure and nature of proteins required for transposition may help to elucidate these phenomena.

J. Davies (University of Wisconsin) and R. Hermann (University of Heidelberg) advocated the use of λ and fd phages respectively as host molecules for studying transposons. Phages possess the distinct advantage in that generally their genetical and physical properties, for example the location of promoters and partial known base sequences, are better characterised than those of plasmids. The ability to synthesise antibiotic-modifying enzymes *in vitro*, described by G. Hogenauer (Sandoz, Vienna) should also help our understanding of the structure and

operation of resistance genes.

A major problem in genetic engineering is the expression of eukaryotic genes in prokaryotes (see *News and Views*, 262, 256; 1976). D. H. Gelfand (University of California, San Francisco) described the construction of plasmids carrying inserted *Xenopus laevis* 28S rDNA "downstream" of the operator-promoter region and part of the β -galactosidase gene of the lactose operon. Induction of the *lac* operon in cells carrying such plasmids causes increased production of RNA complementary to rDNA. Furthermore, some derivatives with inserted genes produce β -galactosidase with reduced enzymic activity but increased molecular weight. The production of polypeptides fused to this enzyme suggests that it may be possible to 'translate' some foreign genes in bacteria.

Potential vectors for natural spread of genes between bacterial genera are plasmids of the P incompatibility group (Datta *et al.*, *J. Bact.*, 108, 1244; 1971). P group plasmids are characterised by an extraordinary ability to transfer themselves to a wide range of Gram negative bacteria. They are thus attractive research tools for studying intergeneric gene transfer (see, for example, *News and Views*, 260, 191; 1976) and for the promotion of chromosomal gene transfer in species which lack their own sex factors. They could in addition contribute to the spread of resistance genes by crossing species barriers and depositing transposons in new hosts. V. Stanisich (University of Bristol) has also found that genes of plasmids normally restricted to *Pseudomonas aeruginosa* can be transferred to and expressed in *E. coli* by a technique essentially involving the formation of genetic hybrids with P group plasmids.

What then confers on these plasmids their unusual capacity for intergeneric transfer? One possibility is that their conjugation mechanism and specificity of attachment of P group sex pili is unaffected by the nature of recipient cells. D. R. Helinski (University of California, San Diego) pointed out that P group plasmids not only exhibit a paucity of cut sites for many restriction endonucleases, but also that their replication machinery may be more complex and hence less dependent on host chromosome function than other plasmids. This could endow them both with some immunity against the battery of restriction enzymes likely to be encountered in intergeneric transfer and with the ability to replicate in a wide range of cellular environments. Accordingly the ability of P group plasmids to cross interspecies barriers in bacteria could well make them mediators of 'natural' genetic engineering. □

Iliopoulos wins his bet

from F. E. Close

The 18th International Conference on High Energy Physics was held in Tbilisi, USSR on July 15-21, 1976

It was the moment of truth for J. Iliopoulos. In his talk at the 17th International Conference on High Energy Physics held in London in 1974 (*Nature*, **250**, 186; 1974) he had declared "I am prepared to bet a whole case of wine that the next conference in this series will be dominated by the discovery of charmed particles". Anyone who accepted the wager should now be preparing shipment because the 18th International Conference, which has just taken place in Tbilisi, USSR, was indeed dominated by that very discovery—the latest in a series of dramatic developments that have revolutionised particle physics in the past two years.

Readers will recall the dramatic breakthrough that occurred in November 1974 when workers at Stanford Linear Accelerator Center, California, and at Brookhaven National Laboratory in New York independently and simultaneously announced the discovery of a heavy metastable meson (called J or ψ) (see *Nature*, **252**, 438, and **254**, 387; 1975). In the decade preceding this discovery all known mesons and baryons that were discovered had fitted in nicely with the idea of Gell-Man and Zweig (*Phys. Lett.*, **8**, 118; 1964) that there should exist quarks coming in three types or "flavours" distinguished by quantum numbers such as isospin or strangeness and that the mesons and baryons were bound states of these quarks. The high mass and remarkable stability of the J/ψ suggested that this meson might be a bound state of a fourth flavour of quark ("charmed quark") and its antiquark. In the jargon this state had "hidden charm" and theoreticians expected that there should be a spectroscopy of similar states which has become known as the "charmonium spectroscopy" by analogy with the spectroscopy of positronium. Several heavy metastable states have been discovered since November 1974 and the latest information on their properties was discussed at Tbilisi. The spectrum that is emerging is in excellent agreement with that expected for charmonium though the precise values of the masses are in some cases not quite what had been theoretically predicted.

The idea that there might be a charmed quark had been suggested by many people as long ago as 1964. Theoretical interest in this idea took on new significance when Glashow, Iliopoulos and Maiani noticed (*Phys. Rev.*, **D2**, 1285; 1970) that a charmed quark could provide a mechanism (the GIM mechanism) for overcoming a stumbling block that had arisen in attempts to unify theoretically weak and electromagnetic interactions. Their theory required that there should exist mesons made from a charmed quark and its antiquark and that the lightest of these mesons should have a mass around 3 GeV/ c^2 (about three times as heavy as the proton). The J/ψ and the other heavy metastable states certainly fit in with this idea and are even in the predicted mass region.

The second consequence of their theory is that there should exist mesons made from a charmed quark and one of the old faithful uncharmed quarks. After the discovery of the J/ψ it was predicted that the lightest of these states ("charmed mesons") should exist with a mass of about 2 GeV/ c^2 . Furthermore it was predicted that strange particles (such as the K meson) should be an important feature of the decay products of these charmed meson states.

In May of this year came a strong suggestion that such a state had been found in a study of the debris produced in the annihilation of electrons with their antimatter (positrons) at SPEAR in Stanford, California. An electrically neutral metastable particle was observed with a mass of 1.86 GeV/ c^2 (compare the predicted 2 GeV/ c^2 !) decaying into K and π and sometimes into K and three pions. A study of the energy and momentum balance suggested that in the electron-positron annihilation a state with mass of 2.02 GeV/ c^2 was being produced in conjunction with the 1.86 GeV/ c^2 state, although the decay modes of this second particle have not yet been isolated. These two states were reported on at Tbilisi and the popular feeling was that they are charmed mesons where the quark and antiquark, from which they are composed, have coupled their spins to give a total angular momentum of 0 or 1 respectively (like π and ρ mesons). The observations of the strange meson, K , in the decay products is as anticipated for a charmed meson.

Only two weeks before the conference physicists at SPEAR working in

collaboration with colleagues from the Lawrence Radiation Laboratory at the University of California, Berkeley, discovered the electrically charged partners of these mesons. The masses of these states are identical to or perhaps marginally heavier than their neutral counterparts. The charged state at 1.86 GeV/ c^2 was seen decaying into a K and two pions. The important discovery, which is crucial to the charm interpretation, is that the positively charmed meson (D^+) is predicted to decay to K and two pions with electrical charges distributed as in

$$D^+ \rightarrow K^- \pi^+ \pi^+$$

but should not decay by the mode

$$D^+ \not\rightarrow K^+ \pi^+ \pi^-$$

This is precisely what is observed in the data presented at Tbilisi.

Other phenomena that are probably due to charmed particle production were reported from several laboratories around the world. Of most immediate comparison with the SPEAR results are the experiments involving electron-positron annihilation at the Hamburg facility (DORIS). B. Wiik (Hamburg) reported that when electrons and positrons annihilate at DORIS with a total energy of just over 4 GeV (the same energy region in which SPEAR saw the simultaneous production of the 1.86 and 2.02 GeV/ c^2 mesons) an electron was observed in the debris more frequently than expected from previously known sources. It was reported last year (see *News and Views*, **257**, 535; 1975) that anomalous production of electrons and muons were seen in this energy range by Perl's group at SPEAR where it was argued that these were the decay products of a heavy lepton. Several features of the DORIS results suggest that at Hamburg, on the contrary, one is seeing electrons produced in the decays of charmed mesons (probably the same mesons that were seen decaying into K and pions at SPEAR). Furthermore, in two independent experiments at DORIS, it has been found that these electrons tend to be produced in conjunction with K mesons. The nature of these events is therefore consistent with pair production of charmed mesons (D, D^*) followed by their "semileptonic" decay

$$e^+e^- \rightarrow D(1.86) + D^*(2.02) \\ \rightarrow e + K + \text{pions} + \text{neutrino}$$

the ubiquitous K meson is as expected in charm theories.

While it seems fairly certain that these leptons are coming from the weak decay of long lived particles, the precise relation of this signal with the anomalous lepton signal observed by Perl's group at SPEAR is unclear and generated much discussion. While the

majority opinion still appeared to be that the Perl group's leptons are coming from a heavy lepton source several people were unhappy with this interpretation.

Now that the first charmed particles have apparently been found, the status of the heavy lepton has taken on a particularly pivotal position in the theoretical development of high energy physics as was emphasised in the talk given by A. de Rujula (Harvard University). As noted above, the priorities of the charmed mesons appear to be in line with those expected by Gashow, Iliopoulos and Maiani if charm is the way to overcome the stumbling block in the weak and electromagnetic unified theories. The discovery of charm with, apparently, these very properties lends indirect support to these theories. Other tests of these ideas were presented at Tbilisi. What seems astonishing is the amount of evidence that has accumulated in the last two years increasingly suggesting that the GIM mechanism allied with the original unification idea of Weinberg (*Phys. Rev. Lett.*, **19**, 1264; 1967) and Salam (in *Elementary particle physics*, edit. by Svartholm, N., 367, Stockholm, 1968) could be, if not the whole story, at least a first qualitative approximation to the correct theory. Almost all of the data on weak interactions reported at the conference agree with the Weinberg-Salam theory with four flavours of quark. There was some discussion as to whether a fifth and sixth flavour of quark may be necessary to describe data (on the theoretical front, confirmation of Perl's heavy lepton would lead people to prefer six flavours of quark—hence its "pivotal position"). There does not yet appear to be any widely accepted need for extra complexity however.

The weak neutral currents (where neutrinos scatter from a target without any transfer of electrical charge taking place), reported on in London in 1974 have been studied in detail. Several groups reported evidence that parity is violated in these interactions, moreover the nature of this violation appears to be in accord with the Weinberg-Salam theory. Beautiful evidence for parity violation in atomic physics was also reported, again in line with the Weinberg-Salam theory.

The consensus of opinion was that it is rapidly becoming accepted that weak and electromagnetic interactions are indeed probably unified and that the "gauge field theories" are underlying not only weak-electromagnetic but perhaps also strong interactions (the new jargon here being quantum chromodynamics, or QCD, analogous to quantum electrodynamics). One consequence of such theories is that the scaling phenomenon seen previously

in deep inelastic scattering of leptons on hadron targets should be violated in a systematic way at the very high energy experiments now taking place at the Fermi National Accelerator Laboratory near Chicago. Experimental data were presented by T. Quirk (University of Oxford) which showed a pattern of scaling violation consistent with the predictions of such theories.

One possibility raised by QCD theories is that quark confinement inside hadrons may be a natural consequence. There was discussion among theorists of this possibility but as yet no clear idea has emerged. Some thoughts on how to proceed were presented by K. Wilson (Cornell University), and one contribution that excited much interest was that of A. Migdal of the Soviet Union who reported his calculations of meson spectroscopy in QCD with some phenomenological success.

When in the future we look back on the two years leading up to this conference, they will surely be seen to rank with the most significant periods of advance in our understanding of subatomic behaviour. A very clear direction for investigation has emerged. With SPEAR having already been perhaps the most productive machine in particle physics history, and DORIS still in its infancy, these electron-positron annihilation machines promise yet more to come. There was also excitement at the great successes in building the new super proton synchrotron at CERN which promises to be ready for experimental research within a few months. East and West at Tbilisi were united in their belief that the next few years in high energy physics may reveal further significant advances that will rank alongside those just made in the past few weeks. I think Iliopoulos would not bet against that. □

Britain in European space

from David W. Hughes

A one day meeting to discuss the future scientific projects to be undertaken by the European Space Agency was held at the Royal Society, London on July 23, 1976 under the sponsorship of the Science Research Council.

DURING the current year ESA will spend £270 million, £200 million of which goes on the space applications programme (which includes Spacelab, communications satellites and the development of Ariane, a three-stage rocket which will make Europe independent of American launch facilities) and £26 million on the science programme. Great Britain contributes about one sixth of this (£6.5 m). The science programme budget is mainly spent on scientific satellites, a typical satellite being funded for five years, the expenditure maximising between the second and third year and totalling in all about £20 million (on average one satellite is launched each year). At present COS-B, GEOS, IUE, ISEE-B and EXOSAT are in orbit or about to be launched. COS-B was launched in August, 1975 and is measuring high energy gamma rays. 1977 is the planned launch year for GEOS which will study particle fluxes and electric and magnetic fields in the outer magnetosphere, IUE, which is an ultraviolet observatory in a geosynchronous orbit (so the satellite is always above the same place as Earth), and ISEE-B which will look at

wave-particle interactions in conjunction with the NASA satellite ISEE-A. It is planned to launch EXOSAT in 1980 to study X-ray sources using lunar occultation techniques. These satellites account for a considerable amount of the projected budget but obviously the amount of uncommitted funds will increase as each satellite comes to fruition, so that in 1978 and beyond there is a considerable amount of money to spend.

Scientific projects in ESA go through a series of stages. First comes the mission definition study in which a group of scientists and space technologists discuss the feasibility of the project in general terms. Then follows a more detailed and searching Phase A study, a Phase B study, construction and launch. Needless to say there is a pyramid structure to this series, the twelve or so mission definition studies per year leading to about three phase A studies and one probable launch.

The main purpose of the London meeting was to canvass the opinions of British space scientists as to what the next main mission should be, what should be flown on the second test launching of the Ariane rocket and which phase A studies should be instigated in 1976/77. With these opinions in mind the Science Research Council will then brief the British delegation for the October meeting of the Science Program Committee of ESA. The fact that the British voice only makes up 17% of the votes makes the dis-

cussion rather academic but democracy being what it is a firm decision by Britain's 17% could sway the day.

Four major missions were discussed, the Space Telescope clearly winning the day and being regarded as an instrument of outstanding and paramount importance. It is hoped that the telescope will appear in NASA's 1978 budget although it has already slipped considerably in time and in size, the main mirror now being 2.4 m in aperture. From its 500–600 km high orbit it will be able to detect objects 100 times fainter than those detectable from the Earth's surface (it will be able to pick up 29th magnitude stars) and will have an angular resolution better than 0.1 arc s, some 10 times better than attainable from the ground. Coupled with this diffraction-limited performance is the fact that being free from atmospheric absorption it will have a spectral range of 912 Å to 1 mm. Within our Galaxy it will study the early and late stages of stellar evolution, the position and proper motion of stars, the dust distribution and hydrogen concentration and also our Solar System. Extragalactic work will include investigations of the structure, scale and evolution of the Universe, the structure and content of galaxies and also quasars. It is obviously a project that no astronomical nation wants to be left out of. NASA and the US Congress look to ESA to contribute more than 10% of the cost in return for an equal percentage of the observing time and an involvement in directing the project. This 10% of the cost would be spent in Europe, designing, developing and constructing some of the instrumentation and the technology (for example the faint object camera and the solar arrays). This cooperation would of course mop up a considerable amount of ESA's science budget for a few years and, while recognising the great importance of the project, some of the scientists present at the meeting pointed out that science *per se* would not suffer if we did not participate, the Americans probably going on by themselves, whereas spending the money on specifically European ideas might open up new horizons in different fields.

The second mission considered was again a cooperation with NASA and involved two spacecraft launched simultaneously towards Jupiter from a space shuttle, and then guided in such a way that one would be deflected by Jupiter towards the north pole of the Sun, the other passing over the south pole. ESA would supply one spacecraft and NASA would use a Pioneer type spacecraft for the other. This "out of ecliptic" mission would provide a new insight into interplanetary and solar physics and the science of the Jovian magnetosphere, mainly by taking the

Aphids, ants and pheromones

from our Insect Physiology Correspondent

It was shown by J. S. Edwards many years ago that the secretion from the cornicles of aphids consists of oily droplets, now known to be a mixture of triglycerides, in aqueous suspension. On release in contact with a solid surface this secretion quickly crystallises to form a hard waxy plaque. It thus provides a mechanical protection against small predators and parasites.

In recent years the cornicles have been found to liberate also alarm pheromones, which are secreted in response to predators and parasites. Bowers *et al.* (*Science*, **177**, 1121; 1972), isolated and identified *trans*- β -farnesene (TBF) as the alarm pheromone in several aphid species; this secretion seems also to repel other aphids encroaching on occupied feeding sites. As with the alarm pheromones of many insects, TBF is interspecific: it is present in all species examined in the subfamily Aphidinae, and all of them (with the exception of the turnip aphid *Hisidaphis*) show alarm activity to pure synthetic TBF. But other unidentified alarm pheromones are present in addition to TBF and these different blends of pheromone probably account for the different responses in different aphid species. Thus the turnip aphid which fails to react to TBF, secretes this pheromone in its cornicle fluid, and it reacts to the secretions of other aphid species.

Nault, Montgomery and Bowers

(*Science*, **192**, 1349; 1976) have now studied the response to these pheromones in the ants which herd certain aphid species. These myrmecophilous aphids enjoying, as they do, protection by their ants from predators, are much less responsive to the alarm pheromones than non-myrmecophilous species. They may walk away on exposure to the alarm pheromone, but they do not drop off the feeding site. At the same time, and not surprisingly, the ants themselves respond to aphid alarm pheromones. In experimental trials it appeared that the ants were prepared to stroke with their antennae any aphid species they were offered; but the different aphid species vary widely in the readiness with which they excrete droplets of honeydew for their ant keepers. Some species will not respond at all. The Macrosiphini were caused to disperse by the ants' stroking; this provoked a predatory response by the ants, and the aphids were carried off to the nest and presumably eaten.

The acceptance of the attentions of ants replaces response to alarm pheromones: the myrmecophilous aphids rely on their ants for protection. Indeed, artificial liberation of TBF leads to attack by the ants, not on the aphids but on any suspected predators by which they appear to be attacked. The threshold of response to the pheromone by the ant is, indeed, even lower than the threshold in the aphids themselves.

observer away from the plane of the planetary orbits where the large majority of observations have been made to date. The two satellites would obtain a stereoscopic view of the Sun and help to test the fundamental symmetry of the Solar System. The basic experiments would consist of a magnetometer, a plasma probe, a solar particle telescope, a cosmic ray telescope, a receiver for studying radio propagation, a coronagraph and a XUV heliograph. The 85° inclination of the orbits to the ecliptic will enable the development of equatorial structures on the Sun to be followed continuously through several solar rotations.

Three other projects are to be studied further. LIRTS, a 2–3 metre classical, uncooled Cassegrain infrared telescope on Spacelab would provide high sensitivity photometry with high spatial resolution enabling many astronomical objects to be studied through the measurement of atomic and molecular lines in the far infrared. EXPOS again uses Spacelab to fly a

set of instruments to study the spectra of cosmic X-ray sources and to detect polarised X-ray emission. The payload comprises a number of large area Bragg spectrometers each selected to study particular features in the 0.5 to 10 keV range. Spatial resolution of about 1 arc min and energy resolution of 10 to 20% at 1 keV is obtained by using an X-ray grating with a large area Walter I grazing incidence mirror. The third project concerned magnetospheric experiments from Spacelab and drew the fascinating picture of a series of tethered subsatellites around Spacelab, like dogs on leads, making multi-point plasma measurements. All these projects have the advantage to the UK that they support research fields in which British scientists are very active and proficient.

The afternoon session of the meeting discussed last year's batch of mission studies and chose the ones that the British scientists would like to go forward for a phase A (feasibility) study. These projects stretched the

imagination much more than the morning's batch as now people were looking towards the space research of the late 1980s.

Space astrometry had an instant appeal, bringing space science and technology to bear on one of the fundamental tasks of astronomy, the observation of the positions of stars and planets as a function of time. The increase in accuracy from ground-based observations has been very limited in the last half-century and no breakthroughs seem to be around the corner. Space astrometry would give positions to between 0.001 and 0.003 arc s (0.04 arc s is the present best for ground-based work); proper motions (the star's velocity perpendicular to the line of sight) could be measured to about ± 0.002 arc s yr⁻¹ after 3 years of observation, such accuracy requiring 50 years at present; parallax (stellar distance) of stars of 11th magnitude, as opposed to 7.5 magnitude at present could be measured to ± 0.001 –0.004 arc s (in contrast to ± 0.013 arc s from the ground). This increased accuracy has enormous astrophysical significance—improving the definition of the cosmic distance scale, calibrating Cepheid variables, increasing the accuracy of stellar luminosity and cluster age estimations being only a few examples to which it might be applied.

The Solar Probe also caused much discussion. Using a Jupiter gravity assist this can get to within 10 solar radii of the Sun's surface. The mission definition scientists decided that the problems of thermal control and telemetry coverage within the corona could be overcome. Scientific objectives are the measurement of the solar gravitational field (J_2), the testing of Einstein's theory of general relativity and the measurement of the rate of change of Newton's constant of gravitation. Also a whole series of experiments dealing with particles and fields in the expanding corona would be flown. A Climatology satellite, to obtain measurements of the Earth's radiation budget and especially a value of the solar constant to $\pm 0.1\%$ (as opposed to $\pm 1.5\%$ at present) was widely welcomed as was a more unusual Dumb-bell Mission. Here two satellites launched into a near polar Earth orbit are deployed so that they are 10 to 20 km apart but tethered together by a long wire. Measurement of the tension variations in the tether gives the gravity field anomalies in the Earth's upper crust. The tether would also be used to measure induced electric fields and the two satellites would be used for active and passive magnetospheric experiments. Finally, an ultraviolet and X-ray satellite (10–1000 Å) with two Baez telescopes and a spectrograph on board was considered.

A year's observation with this satellite would provide a 5 arc min resolution map of the whole sky plus detailed spectra of new sources.

Although one could not help getting the feeling that Britain was a small fish in the ESA pool (which is itself small in comparison to NASA and the USSR's Space Agency) whenever brains combine, new ideas are forthcoming and Britain and Europe can still provide ideas even if they cannot compete in finance and firepower.

Phase changes at 650 km

from Peter J. Smith

It has often been assumed that the Earth's 650-km seismic discontinuity, which involves a large increase in bulk modulus as well as seismic velocity, is largely the result of a phase change in the olivine component of the mantle. Anderson (*Science*, **157**, 1165; 1967), for example, attributed this discontinuity to the disproportionation of spinel to a structure with the properties of mixed oxides—a phase involving the simple oxides, a Sr₂PbO₄ structure or perovskite plus periclase, all of which have similar densities. But as Anderson himself now admits (*Geophys. Res. Lett.*, **3**, 347; 1976), there are problems with this interpretation which suggest that the discontinuity in question may have little to do with transformations in olivine after all.

For example, although the disproportionation of spinel to oxides involves a large increase in density, it also involves an increase in coordination, increasing the average silicon-oxygen separation and reducing the repulsive forces between ions. The increase in bulk modulus is therefore much reduced (and may even be converted into a decrease) compared with that expected from a transformation with no associated change in coordination—a point previously made by Liebermann and Ringwood (*J. geophys. Res.*, **78**, 6926; 1973). In fact, the elastic moduli of spinels and mixed oxides are similar. On this basis, disproportionation as envisaged would be expected to produce a decrease, rather than an increase, in seismic velocity.

Because this is clearly unsatisfactory, Anderson now suggests that the 650-km discontinuity is primarily the result of phase changes in the pyroxene and garnet components of the mantle rather than in the olivine. The basic premise upon which this proposition rests (and detailed evidence for which is promised in a later report) is that the bulk moduli of silicate and oxide solid solutions are almost independent of iron

content. In particular, as Mg²⁺ is replaced by Fe²⁺ in systems such as pyroxenes, olivines and garnets, the density increases in accordance with the Birch formula but the bulk modulus increases or decreases only slightly. The bulk modulus cannot therefore be used to make deductions about the composition of the mantle. It is, on the other hand, a good indicator of crystal structure, its magnitude's increasing from pyroxene to α -olivine to β -spinel to garnet to γ -spinel to oxides in pyroxene proportions to perovskite.

With this in mind, Anderson has attempted to extrapolate the bulk moduli (K) and densities (ρ) of the various regions of the mantle to a pressure of 1 bar and a temperature of 20 °C and has compared the resulting K - ρ points with the K - ρ curves for the appropriate crystal structures. He thus deduces that between depths of 200 and 400 km (Region I) the mantle is predominantly olivine and pyroxene with subordinate garnet, the pyroxene to garnet ratio being 7:2 to satisfy the bulk modulus. Region II, between 400 and 500 km, is close to β -spinel and is consistent with olivine-pyroxene-garnet with a higher proportion of garnet than Region I. Anderson concludes that Region II comprises olivine in the β phase, pyroxene and pyroxene-garnet solid solution with garnet structure.

By contrast, Region III, which lies between 500 and 650 km, is closer to garnet. Its most likely composition is (β + γ)-olivine and pyroxene-garnet solid solution, all the pyroxene now having entered the garnet structure. However, because of uncertainties in the data upon which the K - ρ curves are based, Region III could be entirely β -spinel and garnet. The properties of Region IV (650–800 km) are consistent with this layer's being either mixed oxides or γ -spinel plus stishovite (SiO₂). And finally, Region V, below 800 km, could be either mixed oxides with more stishovite than Region IV or perovskite plus MgO.

In the light of these phase changes Anderson thus proposes that it is the pyroxene-garnet component, transforming from the garnet structure to the mixed oxides or perovskite structure, which is chiefly responsible for the 650-km discontinuity. Unfortunately the thermodynamic data currently available are insufficient to enable the reaction boundary between the mantle pyroxene-garnet solid solution and mixed oxides to be calculated. However, calculations made assuming this complex garnet to behave as almandine-pyrope garnet imply that the transition is sharp (perhaps less than 12 km thick on the basis of admittedly inadequate data and questionable assumptions), a property required by some of the seismic data.

articles

Small-angle scattering experiments on biological materials using synchrotron radiation

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This article describes an alternative approach to small-angle scattering experiments on biological specimens. The method makes use of the unique properties of synchrotron radiation and provides very fast data acquisition with good resolution. Preliminary experiments on rat-tail tendon are described.

UNDERSTANDING the mechanism by which a biological sample responds to a perturbation is a subject of continuing interest and an obvious way of dealing with the problem is to study structural changes by X-ray diffraction. The lattice periodicity in biological specimens is usually larger than that commonly encountered in non-biological crystals and may be as great as 1,000 Å or more. A conventional X-ray tube is normally used to produce characteristic X-ray lines with wavelengths between 0.6 Å and 2.0 Å, and so the first-order diffraction peak will be produced at a small angle to the direction of the incident X-ray beam. As a consequence, the experimenter uses a long camera to achieve reasonable separation of the diffraction pattern from the main beam. Because of the rapid fall-off in intensity with distance from the source, photographic recording of the intensities requires long exposure times, but, in practice, a compromise between spatial resolution and exposure time has to be made. If focusing techniques are used, the exposure times can be shortened but at the expense of complexity in instrument design. Even so, the intensities are still recorded over a long interval.

If one could achieve more rapid data acquisition, then it would be possible to carry out low-angle X-ray diffraction studies of biological material during some temporal process, for instance, muscle contraction. With the technique described here it is possible to record the pattern rapidly and with high resolution using simple apparatus, thus making a dynamic experiment of this type feasible. To do this, we make use of the unusual properties of synchrotron radiation.

Synchrotron radiation has four important characteristics: high intensity, low beam divergence, a high degree of linear polarisation and, probably most important, a smoothly varying white radiation profile¹.

At present, work is in progress to study the applications of this rather special radiation to X-ray diffraction problems. Preliminary work has already been carried out in the field of X-ray topography²⁻⁴ and in powder diffraction (J. B. and A. M. G., unpublished).

Synchrotron radiation

In the synchrotron NINA, at the Science Research Council's Daresbury Laboratory, electrons are accelerated up to 5 GeV and emit electromagnetic radiation which extends as a

continuum from the infrared to the hard X-ray region (about 0.1 Å). The radiation is 100% linearly polarised in the electron orbital plane. The emitted X-ray beam is well collimated, with the photons confined to a cone of half angle between 10^{-3} and 10^{-4} rad. When NINA is operating at 5 GeV with a circulating current of 20 mA, approximately 10^9 photons s^{-1} are predicted at 1 Å within a band width of 0.1% for the apertures used in the experiment (J. H. Poole, private communication). This high flux is particularly valuable for small-angle scattering experiments. Focusing X-ray diffraction cameras have already been constructed and used with synchrotron radiation at DESY (Hamburg), NINA (Daresbury) and SPEAR (Stanford). These cameras use vertically focusing mirrors and curved monochromatising crystals to collect as many photons as possible and, at the same time, to provide small, high contrast diffraction spots on a position-sensitive detector or on a photographic plate.

Here we present an alternative approach to the measurement of X-ray diffraction patterns, which we believe makes good use of the unique characteristics of synchrotron radiation and which does not require any optical components. Furthermore, it allows the very fast collection of data already mentioned. To illustrate the method we have used rat-tail collagen because it combines a large fundamental lattice spacing (670 Å) with well defined, sharp reflections.

Extending energy—dispersive techniques

Our approach to the measurement of X-ray scattering at small angles is conceptually very simple and is an extension of the recent use of energy-dispersive techniques with white radiation from conventional X-ray sources⁵⁻⁸.

The Bragg equation for X-ray diffraction is usually expressed by

$$2d \sin \theta = n\lambda \quad (1)$$

From this it is clear that for a particular spacing, d , and a single X-ray wavelength, λ , the orders $n = 1, 2, \dots$, will appear at the angles 2θ which fulfil equation (1).

If, however, a parallel beam of white (polychromatic) radiation falls on an oriented sample, then for a particular spacing and for a selected scattering angle there will be a number of discrete wavelengths, λ/n , which will satisfy equation (1).

In other words, in place of a conventional X-ray scattering experiment in which the sample is illuminated with monochromatic radiation and the spatial distribution of the scattered radiation is measured, one measures a photon energy spectrum giving all the wavelengths diffracted at a preselected angle.

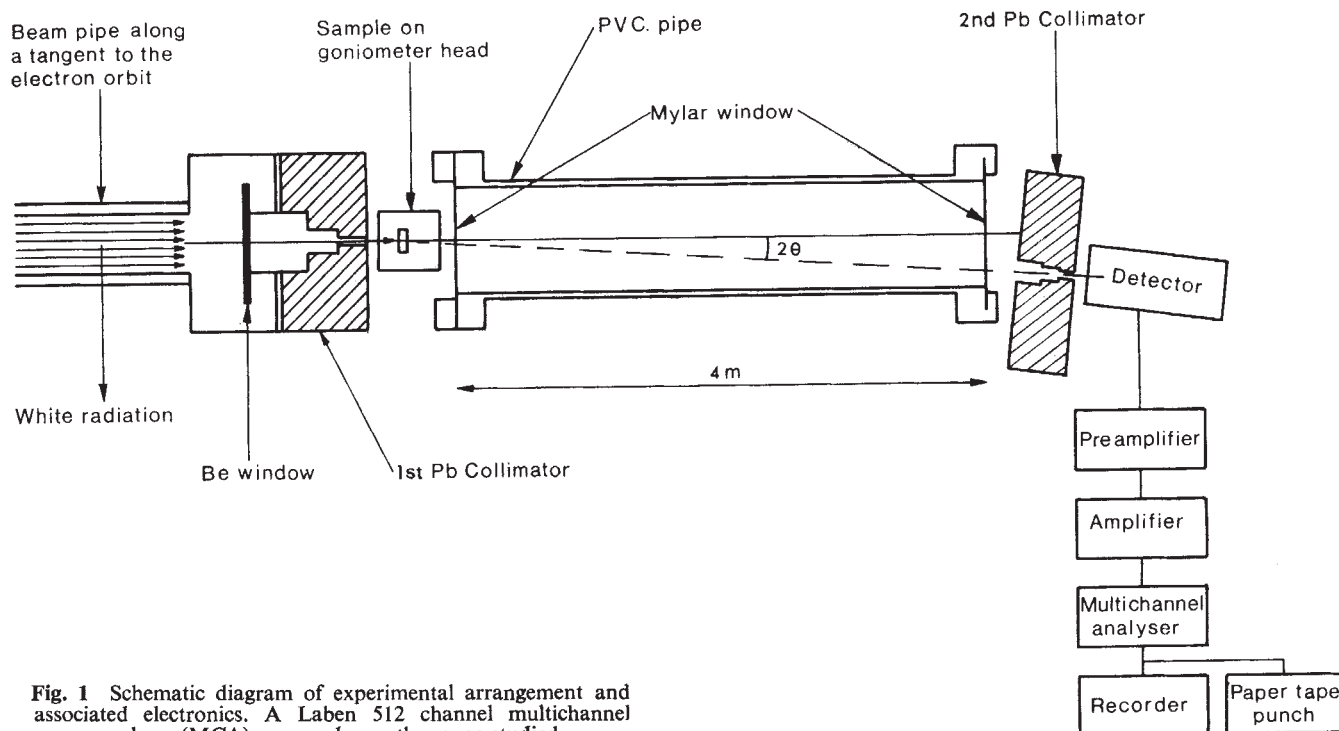


Fig. 1 Schematic diagram of experimental arrangement and associated electronics. A Laben 512 channel multichannel analyser (MCA) was used over the range studied.

We can write the energy, E , in the form

$$E \text{ (keV)} = \frac{12.4}{\lambda \text{ (Å)}} = \frac{12.4n}{2d \sin \theta} \quad (2)$$

Thus, taking θ to be constant we have a strict proportionality between E and n/d . In the normal diffraction experiment, λ is held constant and then $\sin \theta$ is proportional to n/d . Thus, a plot of intensity against energy is similar, qualitatively at least, to the conventional plot against θ . There is a 1:1 spatial correspondence between peaks observed in the two plots for small θ .

The precision of this method relies on the performance of an energy-resolving solid state detector which is commercially available with an energy resolution at best of about 150 eV at 12 keV (information supplied by AERE Harwell and Ortec Ltd). With conventional X-ray sources this technique is handicapped by the complicated X-ray profile comprising white and characteristic wavelengths, as well as the inherent lack of collimation. This means that the measurement of intensities is difficult and, furthermore, in a small-angle scattering experiment high collimation is vital. Most of the uses of energy-dispersive techniques so far have been of a relatively qualitative nature and confined to large scattering angles. In our experiments, a lithium-drifted silicon solid state detector (supplied by AERE Harwell) was used to give a combined resolution for the detector and amplifier system of about 230 eV FWHM, at 12 keV. The time constant of the overall system (25 μ s) could be reduced at the cost of decreasing the energy resolution. The experiments were run at maximum count rates of 1.6×10^4 c.p.s. with a collection efficiency of better than 95%.

Experimental arrangement

A schematic drawing of the apparatus is shown in Fig. 1. White X radiation from circulating electrons in NINA passed down an evacuated pipe, 47 m long, before traversing a 0.016-inch (0.041-cm) beryllium window into air at the end of the pipe. A lead X-ray collimator shaped to minimise any scattered radiation at the detector (mainly from fluorescence of the collimators) was placed in air immediately after the

beryllium window. The sample was supported on a goniometer head and could be rotated within the beam to provide equatorial and meridional reflections at the detector. The collimator aperture was adjusted to suit the size of the specimens and was of diameter 1.0 mm or 0.5 mm. The specimens used were less than 0.25 mm in diameter and 1 mm long.

Immediately behind the sample, a 4-m-long evacuated plastic tube with Mylar end windows was used to eliminate any effects of air scatter or of absorption between the sample and the detector. The angular acceptance of the detector was accurately defined by a second lead collimator placed directly in front of the solid state detector. The main, undiffracted beam was absorbed in a lead beam stop. The scattering angle (2θ) was defined by measuring the distance between the main beam and the detector collimator aperture using X-ray sensitive paper to locate the undiffracted radiation. The entire apparatus was aligned simply and rapidly using a small, portable He/Ne laser. The beryllium windows on the beam pipe and detector restricted the lowest energies available to less than about 3 keV (~ 4 Å). Because of the relatively low counting efficiency of the detector and the source cutoff at high energies, the highest energies accessible were about 40 keV (~ 0.3 Å). The synchrotron was running at 4.6 GeV and approximately 10 mA.

The samples of rat-tail tendon were kept wet during the experiment and were usually stretched, although they were not kept under tension. Orientation of the samples to yield equatorial and meridional reflections was defined by reference to the line joining the centre of the detector collimator aperture to the centre point of the undiffracted beam.

From equation (1)

$$\Delta \theta = \tan \theta (\Delta \lambda / \lambda) = -\tan \theta \frac{\Delta E}{E} \quad (3)$$

and therefore for the particular resolution $\Delta E/E$ of the solid state detector used, the correct angular acceptance $\Delta \theta$ (that is, the appropriate diameter for both collimators) for a particular angle θ was easily calculated. A typical detector collimator would be ~ 0.5 mm in diameter with a separation of aperture from the main beam of from 10 to 50 mm. To calibrate the energy at a particular channel of the multi-channel analyser we

used calibrated X-ray fluorescence lines obtained from commercially available sources. The calibration energies (wavelengths) were those of Cu, Mo, Ag, Rb, Ba and Tb.

Handling the data

In Fig. 2, we plot the raw data from the multi-channel analyser (MCA) display obtained from the meridional diffraction of wet rat-tail tendon. The detector collimator angle (2θ) was set at 3.14 mrad in order to ensure that the first-order diffraction peak fell within the range transmitted by the beryllium window. In the example shown, the prominent first-order peak was already well resolved after only 1 s of data accumulation. A qualitative view of the full spectrum could be obtained within a few seconds but to obtain good statistics data were collected for 200 s. In this experiment, the collimator apertures at the detector and at the sample were chosen to be 1.0 mm and 0.5 mm, respectively. This, combined with the sample size, gave a geometrical resolution comparable with that of the detector. The observed peak widths were twice that expected for the detector resolution and are almost certainly due to the finite size of the aperture collimators. Although the rate of data accumulation could be significantly increased by enlarging the collimator apertures (for a large sample), these experiments are normally limited by count-rate saturation at the detector output.

To make quantitative estimates of the structure factor for each reflection, it is necessary to correct the data in Fig. 2 for the NINA intensity profile, the efficiency of the detector and any other instrumental factors. The first two quantities are, in principle, calculable, but the uncertainty in the remaining correction factors makes it essential to calculate the effect of all of the factors taken together (the total instrument function). Normally, this would be done by using the detector to measure the intensity spectrum of the main beam; but, unfortunately, the detector saturates at a count rate of 10^4 photons s^{-1} , much less than that of a synchrotron source.

There is, however, a neater and simpler method which can be used. The intensity of the background in Fig. 2 arises from incoherent scattering by random atoms, mainly in the air traversed by the X-ray beam, and is given by

$$I = \text{constant} \times (\sum_j f_j)^2 \quad (4)$$

where f_j is the scattering factor for the j th atom. Although it is possible to eliminate this background scatter almost completely, it is better to allow some of this scatter to occur, because it can be used to normalise the diffraction peak intensities. To do this, we note that the value of $\sin\theta/\lambda$ used is extremely small ($\approx 7.5 \times 10^{-3} \text{ \AA}^{-1}$) and corresponds to the flat part of the atomic scattering factor curve (flat to within 1–2% over the range of $\sin\theta/\lambda$ used). Thus, the background intensity, which is a measure of

$$(\sum_j f_j)^2$$

is also a direct representation of the total instrument function. It is then simple to correct for this function by dividing each data point by the background, taking care to extrapolate the background under the diffraction peaks, and then subtracting the number 1.

In Fig. 3 we show the background (total instrument function) on an enlarged scale. It is clear that it rises to a maximum near 7 keV. The resulting normalised intensities of the first- to fifth-order peaks of our sample of rat-tail collagen are shown in Fig. 4. To extend the measurements to higher order peaks, the detector collimator angle (2θ) can be reset to a different angle five times larger than before. This has the effect of shifting fifth-order to the same energy at which first-order diffraction appeared previously. Therefore, if identical synchrotron running conditions are maintained, an angular increase (in 2θ)

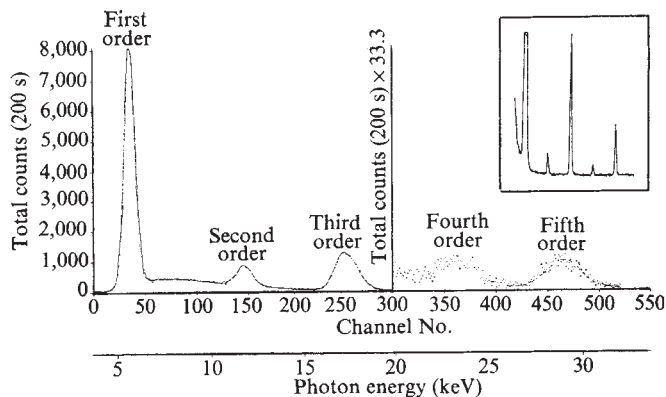


Fig. 2 Raw intensity data from the meridional plane of a wet and stretched rat-tail tendon. Total measuring time, 200 s. Note the low noise level and clean separation of diffracted peaks. Inset, meridional profile obtained with a conventional rotating-anode generator and focusing camera with intensities collected on a photographic film and subsequently densitometered (J. C. Haselgrove, private communication). The sample was approximately $6 \times 0.5 \times 0.5$ mm, camera length 0.65 m, generator at 40 kV, and 60-mA Cu target. Exposure time 30 min.

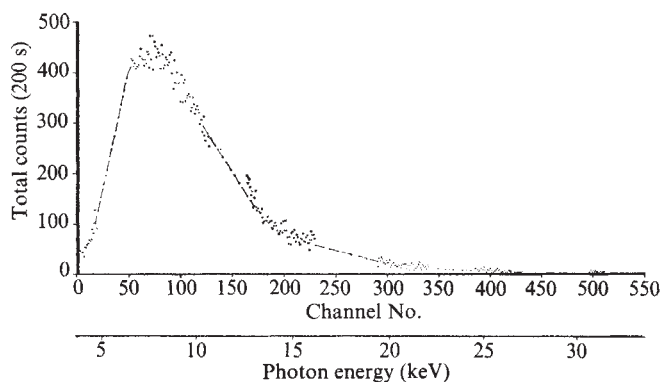
of five times yields the fifth- to the twenty-fifth-order diffraction peaks in place of first- to fifth-order peaks.

The relative intensities of the diffracted peaks given in Fig. 3 agree qualitatively with existing published results⁹ for wet rat-tail tendon.

An example of a profile taken with a conventional rotating-anode X-ray source and a focusing camera is shown in the inset in Fig. 2 and is typical of what can be obtained with conventional sources (J. C. Haselgrove, private communication). The two measured profiles are closely similar: the important difference is in the exposure times and sample size used. The exposure time of the focusing method could be shortened but at a cost of resolution. We note also that in the energy-dispersive experiment all lines are very cleanly resolved with negligible noise. Moreover, the conventional experiment often requires sample preparation involving the mounting and alignment of many collagen strands to achieve sufficiently high intensities. This difficulty is not encountered in our experiment.

Table 1 shows the integrated intensities after the above corrections have been applied. Before we can compare these results with published values, however, it is necessary to take into account the special nature of the energy-dispersive technique. It can be shown¹⁰ that with a white radiation source the integrated diffracted intensity, measured by an energy dispersive system, is a function of λ^2 . Moreover, for small absorption, it depends inversely on the absorption coefficient μ .

Fig. 3 Background intensity profile (total instrument function). — — —, Interpolations under diffraction peaks.



Since to a first approximation $\mu \propto \lambda^3$, the scattered intensity is given by

$$I = \text{constant} \times \frac{|F|^2}{2\lambda \sin^2\theta} \sim \frac{E|F|^2}{2 \sin^2\theta} \quad (5)$$

where E is the energy of the diffracted beam and F is the structure factor. The second column in Table 1 lists the intensities after correcting for this variation. The next two columns list published results⁹ for wet and dry samples and the final column gives our results scaled to the third-order intensity of wet rat-tail collagen.

We find that the intensity of the first-order peak lies well below that measured by Tomlin and Worthington⁹. This probably arises from a combination of effects. In particular, the position of this peak is directly over that region of the background which is changing fastest (Fig. 3), and therefore in dividing by the small background values the resulting estimate of the integrated intensity is subject to a large uncertainty. In addition, this reflection corresponds to a longer X-ray wavelength (2.11 Å) than the other peaks, and, because the scattering efficiency depends on λ^2 , it can be severely affected by extinction. Moreover, the higher absorption at this wavelength will mean that the inverse relationship between I and μ is no longer sufficiently good. One way of getting round these difficulties would be to remeasure the intensity of this peak accurately at a smaller angle. The two measurements thus obtained would allow the absorption effects to be precisely determined. Moreover, if the second angle is chosen to be a third of the present angle, the first-order peak will appear at three times the present energy where absorption becomes less serious.

The intensities of the remaining peaks agree very closely with those measured by Tomlin and Worthington for wet rat-tail collagen. The slightly high value for the second peak may be due to drying of the specimen. In fact, it was observed that the relative intensities of the diffraction peaks changed considerably as a result of drying out of the specimen in agreement with the results of Tomlin and Worthington. Also, in at least one of the muscle samples used, severe radiation damage (a brown spot on the sample) was observed after irradiation for about 20 min. The rapid rate of collection of data means, however, that radiation damage is minimised. The reasonably good agreement between our results for rat-tail collagen and the published intensities suggests that radiation damage is not serious with a collagen sample for the exposure

Table 1 Integrated intensities after application of corrections

n	Integrated intensity (I)	I/E	Tomlin and Worthington Wet	Tomlin and Worthington Dry	Scaled I/E
1	396.1	67.36	1,000	1,000	190
2	52.6	4.47	8	188	13
3	695.9	39.45	111	132	111
4	71.4	3.04	8	49	9
5	307.9	10.47	33	43	30

times used. Experiments with muscle showed that exposures of up to 120 s were feasible with NINA at 5 GeV, 10 mA before any change in the pattern was observed.

Outlook

We have shown that with relatively simple equipment at a synchrotron, it is possible to obtain small-angle scattering patterns from biological specimens which are of extremely high quality and almost noise free. The technique has many advantages over other methods, namely, the high speed of data acquisition, the potential precision of relative intensity measurements, the low level of background radiation and high resolution for material with large spacings.

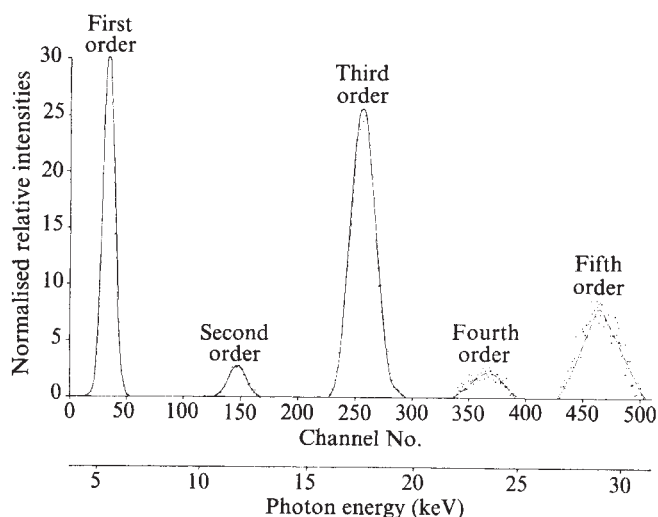
Data can be collected at very high rates using the collimated white radiation from a synchrotron source, and the data acquisition rate is restricted solely by the relatively slow response time of the solid state detector and its associated amplifier. When this limitation is overcome, there could be a further increase in data acquisition rates by several orders of magnitude. Even with presently available electronics we have been able to achieve a four- or fivefold increase in the speed of data collection, simply by using appropriate balanced filters to select a small range of X-ray energies, so that the counter is not saturated by unwanted photons. This allows a larger sample with a larger area of irradiation, and thus more diffracted photons within the energy range selected. Although only one or two reflections can be examined at a time, the advantages of the high speed of collection may compensate for this. When the dedicated storage-ring facility becomes available at the Daresbury Laboratory the X-ray intensities produced will be about 100 times greater than from the existing synchrotron. The storage ring will provide a considerably more stable intensity source, but if full use is to be made of its high intensity output faster energy-dispersive counting devices are needed.

Lattice periodicities of from a few tens of Å to around 1,000 Å can be investigated simply by reducing the detector viewing angle from 10^{-2} rad to a few mrad. A new experimental arrangement under construction at Daresbury will permit the detector to scan through an angular range of 1 to 20 mrad. This could, in principle, permit the observation of up to the 80th order (~ 8 Å) in materials such as rat-tail tendon. Peak-stripping computational techniques together with a high resolution detector should permit peak energy positions to be determined to an accuracy of at least 0.1%. This will offer valuable and complete structure determinations when combined with a two-axis rotation of the sample in the incident beam. Dynamic X-ray diffraction studies should now become feasible if the sample is perturbed periodically and the experiment carried out in the same way as in modulation spectroscopy. For example, an electrical stimulus applied to the sample periodically, with measurements made in and out of phase, would enable a derivative signal to be built up yielding important and new time-dependent structural information on biological specimens.

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Fig. 4 Normalised energy-dispersive pattern from Fig. 2. The basic periodicity is 670 Å.



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Model for action of local anaesthetics

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Sodium channels in nerve membranes are postulated to be surrounded by lipid molecules in the gel (or crystalline) phase. Addition of local anaesthetics triggers a change in the surrounding lipids to the fluid, liquid crystalline phase, allowing the sodium channel to close with resulting local anaesthesia. The experimental evidence for this model is discussed.

DIRECT study of sodium channels in nerve membranes is made exceedingly difficult by their sparsity: it has been estimated that in $1\mu\text{m}^2$ of nerve membrane there are between 2 and 500 sodium channels, depending on the animal¹, compared with about 3×10^6 lipid molecules. Information about the molecular nature of the sodium channel has therefore had to be inferred indirectly, largely from electrophysiological studies. In particular, it has been postulated that the sodium channel contains a narrow slit $3\times 5\text{\AA}$ surrounded by a ring of oxygen atoms, the slit being both the pathway for ion flow and the selectivity filter which determines which ions can flow^{2,3}. I suggest here that the effects of local anaesthetics can be used to probe the lipid environment of the sodium channel.

The block of nervous conduction by local anaesthetics follows largely from a reduction in the sodium conductance⁴, although there are also changes in the time course of the channel gating⁵. The mechanism of this block of sodium conductance has not yet been explained satisfactorily. Since a wide variety of different compounds can act as local anaesthetics, including neutral and both negatively and positively charged molecules, it is not clear whether there is a single mechanism for local anaesthesia, or whether each class of anaesthetic acts in a different way. Staiman and Seeman⁶, however, have shown that effects of anaesthetic mixtures are close to being additive, suggesting a single mode of action.

There are several theories for the action of local anaesthetics. One suggests specific receptors for local anaesthetics in the nerve membrane, and was proposed largely to explain the action of tertiary amine anaesthetics such as procaine, which are most potent in the charged, protonated form and which act only on the inside surface of the nerve membrane⁴. But the receptor (probably the sodium channel itself) would have to be surprisingly unselective, since a very wide range of amines act as anaesthetics, their only obvious similarity being both a lipophilic region and a hydrophilic region⁷. In contrast, very minor changes in the structure of tetrodotoxin, which is known specifically to bind to the outside surface of the sodium channel, cause complete loss of activity⁸. Further, different sites of action would have to be postulated for the action of neutral and negatively charged anaesthetics. A second proposal is that effects of local anaesthetics on the mem-

brane surface charge are important⁴, and although this could account for the action of the positively charged amines, it fails to account for the action of neutral or negatively charged molecules.

The third, and perhaps most popular theory, is the membrane expansion theory. This proposes that anaesthetics increase the fluidity of membrane lipids and expand the membrane⁹. Although anaesthetics have indeed been shown to cause an expansion of erythrocyte membranes¹⁰, it is not clear how an expansion of the nerve membrane would lead to a block of sodium conductance. Further, the theory would predict that an increase in pressure would reverse local anaesthesia, and whereas this is observed for ethanol¹¹, for some other anaesthetics, increasing pressure enhances nerve block¹². Also, local anaesthetics at nerve blocking concentrations would be expected to have little effect on the fluidity of the bulk of the membrane lipids. Thus it has been estimated that the "microviscosity" at the centre of a bilayer of dimyristoyl phosphatidylcholine in the liquid-crystalline state is about equal to that in *n*-decane, and that the viscosity near the glycerol head group of the lipid is only approximately 15 times greater^{13,14}. Since rates of rotation about C–C bonds in short-chain alkanes and alcohols¹⁵ are similar to those at the centre of the bilayer, addition of these alkanes and alcohols would be expected to have only relatively slight effects on membrane viscosity, and yet they are local anaesthetics⁹. Indeed, using a spin-label method, Boggs *et al.* have shown that anaesthetics do not affect order parameters in bilayers of egg lecithin at anaesthetic concentration¹⁶.

The last model for local anaesthetic action is the protein perturbation model, in which interaction between anaesthetics and the sodium channel causes conformational changes resulting in a block of conductance²¹. On such a model it is again surprising that neutral and both positively and negatively charged molecules should act as anaesthetics, especially since the tertiary amines can act in the neutral form, although being more effective when positively charged. The observed competition between Ca^{2+} and the amine anaesthetics is also hard to account for¹⁷, as is the "cutoff" effect (in a homologous series of compounds, anaesthetic potency increases with increasing chain length up to a particular compound, beyond which they cease to be anaesthetics⁹): binding of long chain alcohols to bovine serum albumin simply increases with increasing chain length¹⁸.

A new hypothesis is now presented for the action of local anaesthetics, based on a particular model for the sodium channel.

Annular transition model

The basic postulate of the new model is that the sodium channel is surrounded by an annulus of lipid, which is in the crystalline, or gel, state. If the sodium channel has the

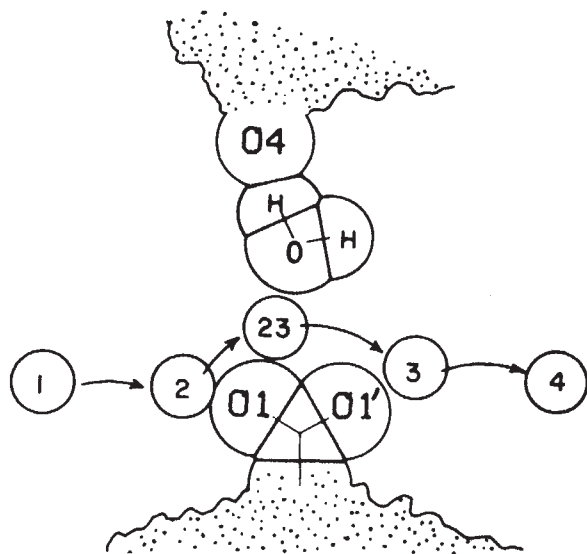


Fig. 1 Model for the sodium channel, with a sodium ion moving through the slit from position 1 to 4 (from Hille, ref. 3).

structure suggested by Hille³, then it is the rigidity of the surrounding lipid microenvironment that keeps the sodium channel open. Addition of anaesthetics triggers a change in the surrounding lipid from the crystalline, gel state to a fluid, liquid-crystalline state. When the surrounding lipid is in a fluid state, the sodium channel is free to relax into its most stable state, with a consequent closing of the oxygen-lined slit which provides the ion channel through the membrane. Figure 1 shows the picture of the sodium channel presented by Hille³, and it can be seen that relaxation of the protein would decrease the distance between the oxygens lining the slit, perhaps allowing hydrogen bonding between the water molecule postulated to be bound in the slit and one of the oxygens O_1 or O_1' of the carboxyl group.

Although the bulk lipids of the biological membrane seem to be free to diffuse within the plane of the membrane¹³, there is strong evidence for relatively long-lived interactions between lipids and at least some membrane proteins. Persistent lipid-protein interactions giving rise to a lipid annulus have been proposed for cytochrome oxidase¹⁹, rhodopsin²⁰ and the calcium transport protein^{21,22}. The model for the sodium channel is, however, unusual in requiring the surrounding lipid to be in the gel phase to allow for activity. For the calcium pump, for example, it has been shown that the enzyme is only active when the surrounding lipid is in the fluid, liquid-crystalline state²³. This presumably arises because in the energy-requiring pumping process, conformational changes occur which are inhibited by rigid, gel phase lipid. In contrast to these relatively slow pumping processes¹³, the rate of passage of Na^+ ions through the open sodium channel is so fast that any change in conformation during ion passage seems unlikely²⁴. Two other membrane proteins have been established to operate within a rigid lipid matrix, bacteriorhodopsin in the purple membrane of *Halobacterium halobium*²⁵ and the microsomal cytochrome P-450 (ref. 26).

Experimental evidence

The fact that anaesthetics do trigger a change in state for lipids from a gel to a liquid-crystalline state has been established in several recent papers. Alcohols up to *n*-octanol have been shown to lower the phase transition temperatures of phosphatidylcholines and phosphatidylethanolamines^{27,28}. The effects are linear with respect to concentration, and the concentrations required to produce

a 5 °C drop in transition temperature are comparable with those required to block nervous conduction⁹. More interestingly, however, it has been found that whereas *n*-decanol lowers the phase transition temperature of dipalmitoyl-phosphatidylcholine (42 °C in the absence of alcohol), it raises that of dimyristoyl-phosphatidylcholine (24 °C in the absence of alcohol). Dodecanol raises the phase transition temperatures of both dimyristoyl- and dipalmitoyl-phosphatidylcholines²⁸. This then is sufficient to explain the "cutoff" effect observed with the alcohols: *n*-nonanol is a local anaesthetic⁹, and it has been reported that dodecanol is less effective than undecanol in suppression of swimming in tadpoles and that tridecanol has no effect²⁹. Similarly, it has been found that the *n*-alkanes up to *n*-octane are anaesthetics, whereas decane is not.

The anaesthetic amines such as procaine and dibucaine also lower the phase transition temperatures of phosphatidylcholines and phosphatidylethanolamines^{30,31}. Now, however, effects are nonlinear with respect to concentration, because of the buildup of charge on the liposomes caused by the binding of the positively charged anaesthetics. This can be overcome by the introduction of negatively charged lipid, which considerably increases the effect of the anaesthetic. When the added negatively charged compound is myristic acid, the effect of the negative charges is masked completely by addition of about 0.5 mM Ca^{2+} or Mg^{2+} (ref. 31). When the negative charge is due to phosphatidylserine, however, there is a differential effect between Ca^{2+} and Mg^{2+} : the effect of the negative charge is removed by about 0.6 mM Ca^{2+} or about 5 mM Mg^{2+} (ref. 30). Differences between the effects of Ca^{2+} and Mg^{2+} on aqueous dispersions of phosphatidylserine have also been found in other studies. Thus the increase in temperature of the phase transition caused by addition of 0.5 mM Ca^{2+} is equal to that caused by 5 mM Mg^{2+} , and whereas Ca^{2+} causes a segregation of phosphatidylserine in mixed bilayers of phosphatidylserine and phosphatidylcholine, Mg^{2+} does not^{32,33}. Furthermore, Hille *et al.* found that Mg^{2+} applied to the outside surface of myelinated nerve fibres had less effect than Ca^{2+} on the voltage dependence of peak sodium permeability, consistent with a weaker binding³⁴. These observations are important because they provide, at least in part, a possible explanation for the fact that the amine anaesthetics are most active when applied to the inside surface of the nerve⁴. Negatively charged lipid has been postulated to be close to the sodium channel in nerve, with the highest concentration on the outside surface. But the Ca^{2+} and Mg^{2+} concentrations of the external medium are about 2 mM and 10 mM, respectively, whereas the Ca^{2+} concentration inside the nerve has been estimated to be micromolar or less³⁵ and the Mg^{2+} concentration is about 3 mM (ref. 36). Thus positively charged anaesthetics will partition most strongly into the inner membrane close to the negatively charged lipid. Similar effects apply to the interactions of chlorpromazine and propranolol (my unpublished observations).

Barbiturates also lower the phase transition temperatures of phosphatidylcholines and phosphatidylethanolamines³¹. Because of the buildup of negative charge, they are most effective in the uncharged form at lower pHs. It has been found that barbiturates are more effective local anaesthetics at low pH, and anaesthetic concentrations are comparable with those required to produce significant changes in lipid phase transition temperature^{31,37}. There seems to be no significant interaction with the negatively charged dipalmitoyl-phosphatidylserine³¹. Connor *et al.*³⁸ have reported that anaesthetic steroids such as alphaxalone also lower lipid phase transition temperatures.

There are several other experimentally testable consequences of the model. First, if anaesthesia results as a consequence of a lipid transition from the gel to the liquid-crystalline state, then a decrease in temperature should restore the lipid to the gel state and consequently reverse

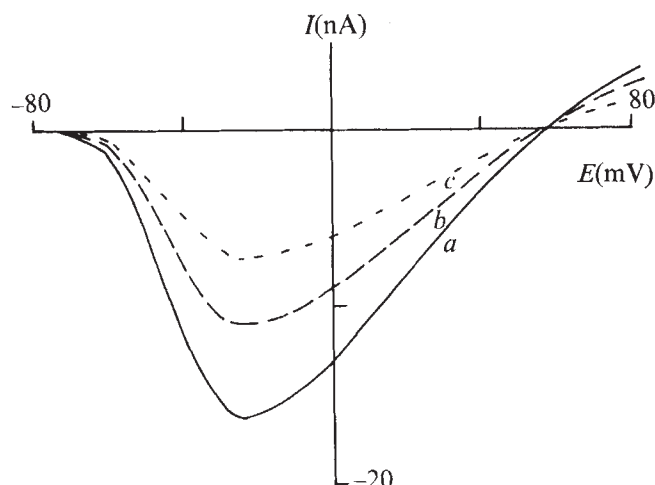


Fig. 2 Peak current (I)–voltage (E) relations as a function of the height of the selectivity barrier. Internal Na^+ , 11.2 mM; external Na^+ , 116 mM; T , 5 °C; curve a , barrier height $G23 = 5$ kcalorie mol^{-1} ; curve b , $G23 = 5.26$ kcalorie mol^{-1} ; curve c , $G23 = 5.53$ kcalorie mol^{-1} .

anaesthesia. Only one experiment of this kind seems to have been reported: Spyropoulos did indeed find that the anaesthetic effect of ethanol on squid axon at 22 °C was reversed completely by lowering the temperature to 4 °C (ref. 11) (in the absence of a lipid phase transition, the partition coefficient for small hydrophobic molecules between lipid and aqueous phases changes only slightly in this temperature range³⁹). A second consequence is that the sodium conductance of nerve should be little affected by a decrease in temperature from the acclimatisation temperature of the animal (until a temperature is reached at which the gating mechanisms are affected), whereas an increase in temperature should lead to loss of conductance. Unfortunately, the appropriate experiments do not seem to have been performed. Moore⁴⁰, however, has observed that the sodium conductance of squid axon is a linear function of temperature within the range 5–25 °C with a temperature coefficient only slightly higher than that for electrolyte conductance in water: this contrasts with the very sharp drop in the activity of membrane-bound proteins such as the Na^+ - K^+ -ATPase with decreasing temperature. Interpretation of the effects of temperature on nervous conduction are likely to be complex, because of changes in rate constants and conductances for both the sodium and the potassium channels. Nevertheless, with frog nerve the spike amplitude increases slightly on cooling from 25 °C to 8 °C, even though spin-label studies have shown a considerable decrease in fluidity for the bulk lipids within the same temperature range^{41,42}. In contrast, increasing temperature produces a reversible conduction block, the temperature of block depending on the animal^{43,44}. Thus the available evidence certainly does not contradict the hypothesis of a rigid microenvironment for the sodium channel. Even if more detailed data were available on the effects of temperature on sodium conductance, their interpretation would likely be difficult because of the hysteresis effects expected in such a system. In fact Inoue *et al.*⁴⁵ have found marked hysteresis in the effects of temperature on the membrane potential of squid axon internally perfused with NaF and bathed in CaCl_2 and NaCl.

Increased pressure is predicted to have two distinct, and contradictory, effects. First, it has been found that increased pressure increases the temperature of a lipid phase transition, simply because a lipid in the liquid-crystalline state occupies a larger volume than one in the gel state⁴⁶. Initially, therefore, an increase in pressure would be expected to inhibit the effect of an anaesthetic. Once the anaesthetic

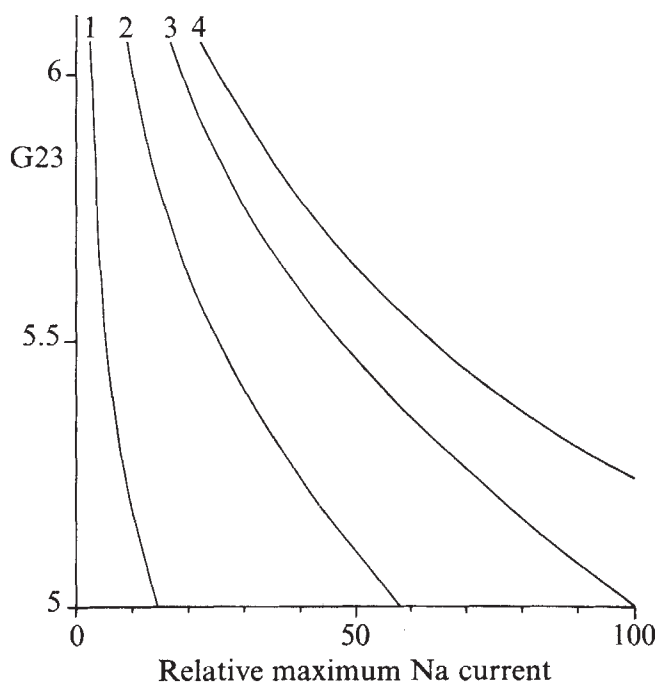
has caused the lipid to transform from the incompressible gel state to the compressible liquid-crystalline state, however, an increase in pressure would be expected to increase the relaxation of the protein (since a relaxed protein with a partially closed sodium channel occupies a smaller volume than that with a fully open channel) and so enhance the effect of the anaesthetic. Both inhibition and enhancement have been observed experimentally^{11,12}.

Simulation of anaesthetic effects

Although relaxation of the sodium channel caused by anaesthetics could cause blocking of the passage of sodium ions at any point along the length of the channel, the most likely point of blockage in Hille's model³ is in the narrowest region of the channel, referred to as the selectivity filter. The size and molecular nature of this filter was chosen so that passage of Na^+ through the filter should be favoured over any other ions, whether larger or smaller than Na^+ . Hille³ has presented a four-barrier model for the sodium channel, with the largest of the barriers corresponding to this selectivity filter. It is interesting therefore to see how well the effects of local anaesthetics can be simulated by this four-barrier model, assuming that the relaxation of the protein affects only the height of the barrier constituting the selectivity filter. The following results were obtained using the equations for the one-ion pore model as presented by Hille, and the parameters for the energy profile found by Hille to give the best fit to the ion-selectivity of the sodium channel of frog myelinated nerve. Figure 2 shows the effect of varying the peak height of the selectivity filter ($G23$ in Hille's notation) on the peak current–voltage relations. The effect clearly is to reduce the sodium current, with no effect on the reversal potential or on the position of maximum sodium current. This is what is observed for the action of neutral anaesthetics such as ethanol⁴⁷, although charged anaesthetics sometimes cause shifts along the voltage axis.

As Fig. 3 shows, effects of increasing barrier height on

Fig. 3 Effect of external sodium ion concentration on peak sodium current as a function of barrier height $G23$ (kcalorie mol^{-1}), with internal 11.2 mM Na^+ , 5 °C. The data are normalised to 100 for peak current with external 116 mM Na^+ and $G23 = 5$ kcalorie mol^{-1} . External Na^+ concentration: 1, 14.5 mM; 2, 58 mM; 3, 166 mM; 4, 200 mM.



peak sodium current gradually decrease. Thus the expected dose-response curve would be hyperbolic. The anaesthetic would have no effect until its concentration was sufficiently high to trigger a lipid phase transition for some of the lipid surrounding the sodium channel. This would make possible a partial relaxation of the channel, with an increase in the height of the energy barrier of the selectivity filter, and a corresponding large effect on sodium conductance. Increasing anaesthetic concentration would increase the proportion of surrounding lipid in the liquid-crystalline state and further increase the height of the energy barrier, but with a gradually lessening effect on sodium conductance. Although no detailed comparison is possible, published dose-response curves have this general form^{48,49}. The data for Fig. 3 also show that the effect of changing the barrier height is more marked at high external sodium concentration than at low, although complete block of the sodium current occurs first at lower sodium concentrations. This latter observation has been confirmed experimentally by Condouris⁵⁰.

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Dietary practices and growth responses as predictors of longevity

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The longevity of rats permitted freedom of dietary choice can be predicted accurately solely on the basis of their dietary behaviour and growth responses early in life.

CHRONIC underfeeding of a complete diet is the only means known for increasing the length of life of homoeothermic laboratory animals beyond the limits characteristic for the species¹⁻⁸. At the other extreme, chronic overfeeding, or other dietary excesses or imbalances, curtails lifespan^{2-5,9,10}. For animals allowed freedom of dietary choice, a mode of feeding that approximates natural conditions, longevity correlates highly with both the amount and type of diet an animal elects to consume before mid-life¹¹. Dietary practices during early life are particularly important. The quantity and composition of the diet determines in large part the progression of body weight changes throughout life. Growth rate and other body weight-related expressions should therefore also correlate with longevity^{1,2,8,12-16}. We show here that there are, in fact, several parameters involving body weight that correlate more closely with lifespan than any of the dietary variables.

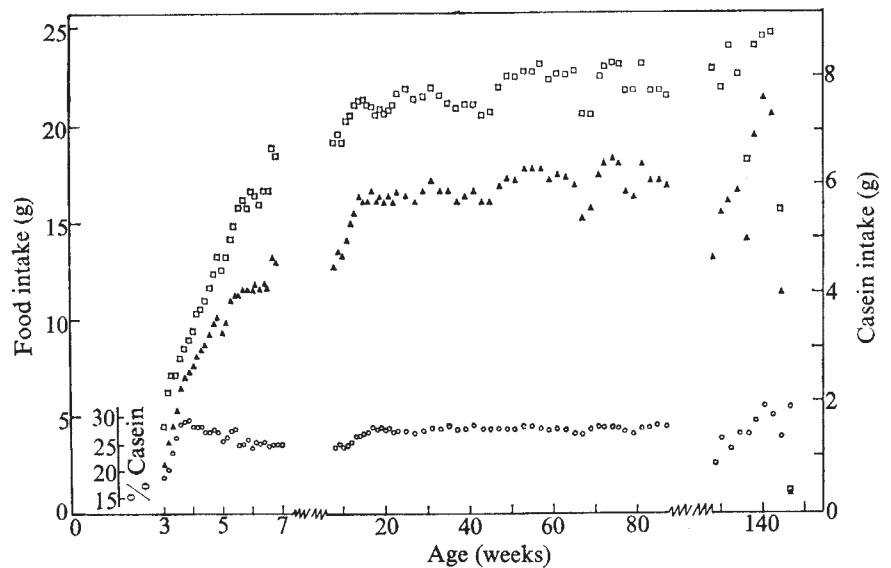
The dietary practices, body weight changes and duration

of life of rats fed on a self-selection basis differ from individual to individual. In assessing the extent to which an animal's lifespan is directly linked with its dietary and growth behaviour, we began the analyses at a stage similar to that used by others working with fixed dietary regimens. Each lifespan-related trait was thus considered separately, but the variety and interdependence among these traits require a more complex treatment to understand their synergistic effects. Here, therefore, we have stressed the multiple variable approach for forecasting lifespan. We show that regardless of the proximate cause of death, there are combinations of specific conditions early in life which allow accurate prediction of an individual's lifespan.

Dietary and growth traits

Individually housed male Charles River COBS-CD rats were offered daily, throughout postweaning life, three complete purified isocaloric diets¹⁷ in separate containers. The diets differed only in their protein and carbohydrate content. To ensure that each of the 121 rats in this study had sufficient freedom to exhibit its individual preference, the proportions of the protein and carbohydrate components of the diets

Fig. 1 Average food and protein intake of rats fed on a self-selection basis throughout life. $\square$, Mean daily food intake; $\blacktriangle$, mean daily protein (casein) intake; $\circ$, proportion of protein in the composite diet selected by the rats.



were set at 10 and 70.5%, 22 and 58.5%, and 51 and 29.5%. Vitamin-free casein, sucrose and corn oil (13.5%) were the sources of protein, carbohydrate and fat, respectively. The adequacy of the mineral (6%), vitamin and trace element content has been demonstrated earlier with conventionally fed animals¹⁷⁻¹⁹. The dietary history during gestation and suckling and the critical maintenance of constant environmental conditions have been described¹⁷.

The absolute and relative intake of the diets differed; consequently, each rat selected a diet differing in quantity and composition. In general, after an initial rapid increase in food intake (Fig. 1), the daily amount consumed remained relatively constant for a prolonged period. This phase persisted, for most of the rats, for as least 30 weeks.

The protein-carbohydrate content of the composite diet selected by some rats changed with age. A change in preference, if it occurred, was generally to diets of lower protein content and was detectable by the end of week 2 or 3 of feeding. At approximately 8 weeks of age, this trend reversed so that the diets subsequently chosen contained progressively higher percentages of protein. Beyond 4 months of age, the average protein content of the diet

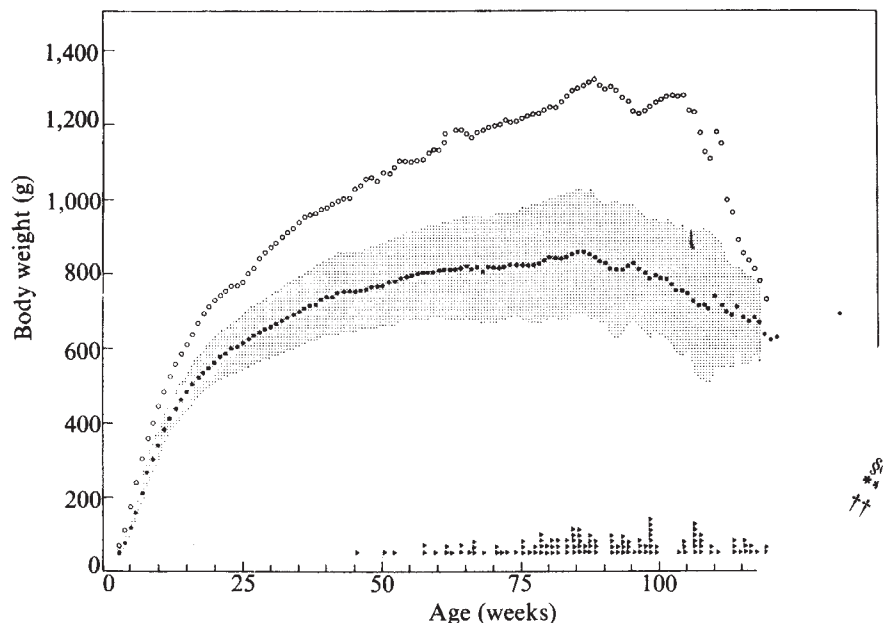
selected by some of these rats generally exceeded that preferred by rats whose preference had more or less stabilised within the first month. For the entire population, however, the average proportion of protein remained uniform throughout the greater part of life (Fig. 1).

The rats were weighed weekly, when they were also examined for gross signs of disease. No therapeutic measures were undertaken except for shortening excessively long incisors. The presence, type and severity of disease were based on histopathological assessment. These results have previously been reported²⁰.

The average changes in body weight with age are shown in Fig. 2. No two rats had identical rates of growth and durations of the growth period. The differences among the rats were further accentuated when body weight and changes in body weight were related to gross efficiency of food utilisation¹⁸ and the quantity of food, or of protein, consumed relative to body weight.

The heavier the rat, the more food it tended to eat but, relative to body weight, the less food it consumed. The latter is explained by the fact that the heavier rats were generally more efficient than lighter, slower growing rats in

Fig. 2 Growth pattern of rats fed on a self-selection basis throughout life. $\bullet$, Mean body weight (shaded area represents 1 s.d. above and one below the mean); $\circ$, heaviest weight attained by any individual; $\blacktriangleright$, age at death of a rat. Irregularities in weight curves due to weight loss that generally precedes death.



converting what they consumed into body mass. The heavier rats thus tended to gain more weight than would be expected on the basis of food intake alone.

Correlation of individual variables with lifespan

The mean lifespan (632 ± 125 d), population half life, age when most deaths occurred, and life expectancy²¹ were in close agreement. The frequency distribution curve for age at death was nearly symmetrical and the age-specific mortality rates increased in a near-exponential manner. The age at death of the shortest lived rat was 314 d and the longest was 1,026 d (Fig. 2).

The correlation data are summarised in Table 1. Additional details on the relationships among lifespan and dietary practices have been presented elsewhere¹². For most variables, significant associations were found relatively early in life and in all cases, before mid-life. In addition to differences in age when a correlation was first detected, the duration, direction and magnitude also differed. In general, the body weight variables correlated more closely with lifespan than did the dietary variables. The highest simple correlation values were obtained when lifespan was related to the number of days needed by the rat to reach a pre-assigned weight (Table 1). Of all the variables examined, only the maximum body weight attained during maturity did not correlate significantly with lifespan.

Combinatorial effects of diet and weight-related variables on lifespan

The simple correlation values presented in Table 1 represent only associations between pairs of variables; they do not imply causality. Furthermore, the interdependence among the parameters (Table 2) precludes ascribing to an isolated factor a direct connection to length of life. The correlation

may be indirect, in that a variable might be closely associated with another factor that is directly related to lifespan. Since each variable has a unique period when it correlates with lifespan, there are added difficulties in interpreting simple correlation data. It is necessary, therefore, to determine how a number of variables interact and this can be best accomplished by construction of a model. If the mathematical model is accurate, it shows a dependence relationship and thereby allows the estimation of lifespan. Since countless factors can affect length of life, however, it would not be realistic to expect that the lifespan estimated from a mathematical model, based on a limited number of variables, would exactly match the observed lifespan. Deviations of the estimated lifespan from those observed represent errors; the smaller the error, the better a model explains the longevity data. Since on a simple correlation basis, nearly all the variables relate significantly with lifespan, model building was used to reveal the combination of factors that best explain the lifespan data. We applied the methods of Furnival and Wilson²² to our problem and their usefulness lies in the fact that they minimise the total squared error²³. When the best combination was identified, the relative importance of each variable was then determined by the least squares method²³ and expressed in terms of a multiple regression equation. In no case was a model acceptable unless the contribution of each factor was statistically significant ($P < 0.05$). To compare models, the proportion of variance explained (R^2) was useful, in that it describes the extent to which the errors have been minimised.

The differences in length of life among the animals were best explained by a model that incorporated seven factors (see equation *a* in Table 3; the correlation matrix is given in Table 2). Four of these variables characterised the growth pattern and the remaining three defined the preference for protein and the efficiency with which the amount of food consumed is utilised for growth. Deletion of any one of

Table 1 Summary of simple lifespan correlation with dietary and body weight-related variables

Variable	Significant association (age and body weight limits)		Maximum correlation coefficient*	
	(d)	(g)	(r)	(P)
Food intake†(g)	49–372		–0.40	< 0.00001
Non-protein calorie intake†	49–309		–0.39	< 0.0001
Carbohydrate intake†(g)	49–299		–0.36	< 0.0001
Protein intake†(g)	43–49		0.18	< 0.05
	200–499		–0.27	< 0.01
Protein content of diet‡(%)	43–49		0.18	< 0.05
	300–499		–0.21	< 0.05
Body weight‡(g)	63–406		–0.43	< 0.00001
Weight gain (g)	49–294		–0.44	< 0.000001
Relative growth rates ¶	49–147		–0.28	< 0.01
Maximum body weight attained (g)	—	—	–0.07	< NS
Age to attain specified weight§(d)		300–600	0.46	< 0.000001
Age-independent body weight acquisition time**(d)				
for 100-g increments		200–300 to 500–600	0.48	< 0.000001
for body-weight doubling		200–400 to 300–600	0.49	< 0.0000001
Food intake per kg body weight††(g)	148–294		0.23	< 0.01
Protein intake per kg body weight‡‡(g)	43–49		0.22	< 0.05
Gain in weight, g per total food intake(g)	49–196		–0.29	< 0.001

* $n = 120$ or 121 .

†Average daily intake for successive weekly intervals.

‡Computed for successive weekly intervals.

|| Computed for successive 7-week intervals.

¶ Instantaneous relative growth rate (K)

$$= \frac{\log_e \text{weight}_2 - \log_e \text{weight}_1}{t_2 - t_1}; t_2 - t_1 = \text{age in weeks}$$

Computed for successive 100-g intervals.

§ Age at weight_2 – age weight_1 .

Value for each period represents the mean of a series of 7 successive weekly values:

$$\Sigma \left(\frac{\text{average daily intake for 1 week}}{\text{body weight at end of week}} \right) / 7$$

Table 2 Relationships between diet, body weight and lifespan

	Correlation matrix (<i>r</i>)*									
	Body weight† 119 d	Body weight† 133 d	Time to double weight‡	Age to attain specified weight	Daily food intake¶	Feed efficiency§	Protein intake**	Protein content of diet††	Protein intake per kg body weight¶¶	Length of life
Body weight at 119 d	1.000									
Body weight at 133 d	0.987	1.000								
Time to double weight‡	-0.755	-0.759	1.000							
Age to attain specified weight	-0.782	-0.777	0.992	1.000						
Daily food intake¶	0.751	0.790	-0.493	-0.485	1.000					
Feed efficiency§	0.438	0.466	-0.614	-0.558	0.211	1.000				
Protein intake**	0.300	0.269	-0.195	-0.255	0.150	-0.146	1.000			
Protein content of diet††	0.204	0.182	-0.167	-0.213	0.059	-0.006	0.924	1.000		
Protein intake per kg body weight¶¶	0.096	0.086	-0.120	-0.143	0.063	-0.018	0.900	0.922	1.000	
Length of life	-0.460	-0.455	0.481	0.455	-0.428	-0.331	0.226	0.216	0.255	1.000

*Level of significance (*P*) of correlation coefficient values (*r*); *r* values 0.18, not significant; *r* values 0.18–0.22, 0.23–0.28, 0.29–0.33, 0.34–0.39, . . . , correspond to *P* < 0.05, < 0.01, < 0.001, < 0.0001, . . . , respectively. One rat did not attain 500 g and protein intake data for the 43–49-d period was not available for another because of excess spillage of food; correlation matrix and multiple regression analysis therefore based on *n* = 119.

†Body weight at 119 d, mean = 522 g (range: 298–665); at 133 d, mean = 549 g (range: 312–708).

‡Time (d) to double initial body weight of 250 g; mean = 64.2 (range: 33–399).

||Age of animal (d) when it reached 500 g body weight; mean = 119 (range: 83–494).

¶Average daily food intake (g) between 14 and 21 weeks of age; mean = 21.0 (range: 15.5–26.7).

§ $\frac{\text{Body weight at 98 d} - \text{body weight at 49 d}}{\text{Total food intake (g) for same period}}$; mean = 0.26 (range: 0.13–0.31)

**Average daily protein intake (g) between 43 and 49 d of age; mean = 4.2 (range: 1.4–9.6).

††Average daily protein content of composite diet (%) selected between 43 and 49 d of age; mean = 24.4 (range: 10.1–49.2).

¶¶Average daily intake (g) between 43 and 49 d per kg body weight at 49 d; mean = 19.6 (range: 9–40).

these variables, or the substitution of any parameter with data representing other age or time periods, or weight changes, led to a less informative multiple regression equation. The addition of other variables to the model or logarithmic transformations of the data did not improve the fit either.

The seven variables acting in concert, accounted for 46% of the variance in the length of life (Table 3). In contrast, on the basis of simple correlation analysis, no single variable explained more than 24% of the variance. The data given in Table 3 show that the changes in body weight occurring long before the growth pattern had been completed accounted for the greater proportion of the explained variance in the duration of life.

The predictive capability of the multiple regression equation was evaluated by comparing the estimated length of life with the actual length of life. This form of analysis also served to identify major sources of error in the predictions. The average absolute error of 12% from the actual number of days lived indicated that, at the very least, a rat's lifespan can be predicted as being of short, intermediate or long duration.

An additional series of analyses were made to determine to what extent the elimination of several of the shortest lived rats would improve the model for the others. Only those rats that lived longer than 425 d, representing 95% of the population, were used to derive the 'conditional' regression equation *b* (Table 3). The small changes in the coefficient values from *a* to *b* resulted in estimated lifespans that more closely approximated actual lifespans. The increase from 46 to 52% in the proportion of variance explained was greater than would have been obtained by the application of equation *a* to the same animals.

These observations suggested that as the number of animals in the population decreased because of attrition, a prospective, step-wise redefining of the best association between the independent variables and lifespan for the survivors would increase the accuracy of lifespan estimate. This was confirmed as indicated by the conditional multiple regression equations *c* to *f* (Table 3).

The most precise estimate of lifespan was obtained for rats that survived more than 700 d (equation *f*), where the average absolute error was less than 4% and, moreover, only four variables were needed. With the exception of this subset of animals, the progressive reduction in the absolute error with the other conditional regression equations was not due to an increase in the degree to which the specific combination of variables correlated with lifespan. Rather, the apparent increase in accuracy was the result of reductions in the number of cases of extreme over- and under-estimates. These adjustments, however, were not as critical as changes in the type and relative importance of the variables comprising the series of conditional regression equations. Thus, the information required to estimate lifespan of all the rats was not identical to that needed for a segment of the population. The contribution of dietary preference and food efficiency data became progressively less important as the number of short and intermediate lived animals included in a cohort decreased. Body weight-related variables, however, took on greater importance (Table 3). Thus, those animals that choose and efficiently utilise early in life a low-protein but otherwise adequate diet, and that complete the greater part of their growth by the time they are approximately 100 d old, will probably be the shortest lived members of the population. At the other extreme, rats that grow slowly, particularly between 50 and 150 d of age, will more likely have extended lifespans.

In view of the lifespan-dependent changes in the number and type of variables, a single all inclusive equation cannot be expected to describe adequately the length of life of all the rats. At best it represents a compromise. The under-estimation of lifespan for long lived rats obtained when equation *a* was used, was partly the result of having included dietary variables. At the other extreme, the overestimation for the short lived rats probably resulted from inclusion of body weight variables. A retrospective analysis confined to the shortest lived rats (25% of the population) confirmed that the weight of the rat *per se* was not needed to estimate lifespan. The protein intake was replaced by the amount of carbohydrate consumed, but the age to reach 500 g and

Table 3 Estimation of individual lifespan as determined from growth and dietary practices early in life (conditional multiple regression analysis)

Coefficients of linear regression equation for estimating lifespan*											
	Fraction of population (%)	Range in lifespan (d)	Cohort size (no.)	Body weight 119 d (g)	Body weight 133 d (g)	Age at 500 g (d)	Body weight doubling time† (d)	Gross food efficiency†	Protein intake† (g)	Protein fraction of diet† (%)	Protein intake per kg body weight† (g)
(a)‡	100	317-1,026	119	-3.54	2.13	-7.51	9.41	1,634	59.90	-6.24	
(b)	95	425-1,026	113	-3.69	2.24	-7.84	9.71	1,820	56.76	-6.62	
(c)	85	500-1,026	101	-3.54	2.30	-6.36	8.00	1,389	51.96	-6.19	
(d)	70	575-1,026	83	-2.54	1.69	-5.27	6.66	747	14.26		
(e)	50	620-1,026	60	-2.75	2.28	-4.72	5.92				2.58
(f)	25	700-1,026	30	-3.87	3.54	-4.64	5.73				

Statistical summary								
Estimated compared with actual lifespan				Fraction of variance explained by				Contribution of weight factors to lifespan (%)§
Fraction of population (%)	Correlation coefficient (r)	Mean standard deviation (d)	Absolute average error (%)	Weight + dietary factors (R²)¶	Weight factors only (R _w ²)¶	Dietary factors only (R _d ²)		
(a)‡	100	0.680	±95	12.1	0.462	0.340	0.159	74
(b)	95	0.722	±81	10.2	0.521	0.400	0.152	77
(c)	85	0.711	±73	8.8	0.506	0.392	0.170	77
(d)	70	0.688	±68	7.5	0.473	0.405	0.169	86
(e)	50	0.691	±60	6.2	0.477	0.427	0.023	90
(f)	25	0.815	±43	3.8	0.665	0.665		100

*Coefficients for the linear regression equation of the form: Estimated lifespan = $-c_1$ (body weight at 119 d) + c_2 (body weight at 133 d) $-c_3$ (age at 500 g) + c_4 (body weight doubling time) + c_5 (gross food efficiency) + c_6 (protein intake) $-c_7$ (protein fraction of diet) + c_8 (protein intake per kg body weight). Blank entry implies that the variable is not necessary in the equation.

Intercept coefficient $\pm$ s.d. for equations a, b, c, d, e and f: 1,085 $\pm$ 227, 1,113 $\pm$ 196, 1,065 $\pm$ 185, 1,026 $\pm$ 187, 1,027 $\pm$ 207 and 1,031 $\pm$ 214, respectively.

†See footnotes of Table 2 for source of data and range of the values.

‡Equation designation.

||All *F* values significant at $P < 0.0001$.

¶ R^2 , coefficient of determination (proportion of variance explained).

§(R_w^2/R^2)100.

the time to double a body weight of 250 g were still significant lifespan predictors.

The sequential redefinition of the best combination of variables, therefore, afforded the only available means of improving the lifespan forecast over that obtained with a single mathematical model. The weighted, overall mean standard deviation from the actual lifespan determined by the step-wise application of the appropriate regression equation to each of the rats within a cohort, was ± 69 d. This corresponded to an absolute error of 8% and the proportion of the variance explained approached 50%.

Comparison of fixed and self-selection regimens

In experimental studies using fixed diets, the changes in life expectancy and the frequency of age-related diseases attributable to chronic severe under-nutrition and excessive over-nutrition have stressed the influence of the quantity of food consumed¹⁻¹⁰. Deviations in life expectancy and disease frequency that may occur among animals maintained on fixed diets differing in composition have also been interpreted to result in part from changes in appetite and therefore in intake of food or calories⁷. These changes may accentuate or obscure the effects of a dietary component. With self-selection feeding, food intake data *per se* are not necessary to forecast duration of life. Useful information is however obtained from data which describe the composition of the diet, the efficiency with which it is used for growth purposes, and the manner in which age-dependent and age-independent changes in body weight occur. The omission of caloric intake as an important factor which influences the rate of ageing should not be considered to be at variance with earlier conclusions. Rather, it indicates that the inter-

actions among lifespan-related factors provide other combinations of variables that are more revealing of future events. Some of these variables may be influenced by food intake. The absolute changes in body weights and time required to attain a specific weight act as substitute information for food intake. Body weight, which as an isolated variable correlated even more closely with lifespan than food intake, is also unnecessary, since these data are also incorporated in the absolute body weight changes and the time required to attain a specific weight.

A fixed diet high in protein and low in carbohydrate, when maintained throughout life, is associated with a higher life expectancy compared with that obtained with diets containing relatively lower levels of protein^{17,24-26}. With self-selection feeding, the proportion of protein in the diet early in life also correlates directly with lifespan, but later, the opposite condition prevails¹¹. Only the protein-carbohydrate ratio of the diet selected early in life seems to be relevant in the context of the other variables. In the multiple regression equations a-c, however, the coefficient values for this variable are negative. This does not indicate that a lower proportion of protein in the diet implies a longer lifespan. Instead the negative value serves to moderate the effects on lifespan contributed by the absolute amount of protein consumed. Thus, apart from the influence of the other variables, the magnitude of the life-shortening effect resulting from a low protein intake early in life is accentuated or minimised depending on the relative amount of protein in the diet selected. Analogous feedback systems are evident among the two pairs of variables in equation a that describe growth. In both cases, one member of a pair exerts an influence opposite, but not of equal force, to that supplied by the other.

Since dietary intervention has been shown to modify longevity, regimens designed to conform with the dietary practices of long lived rats, may be effective in increasing lifespan. The extent to which this type of dietary manipulation would be successful depends on the interactions among the specificity in dietary requirements of the individual, the stage of life when the new regimen is begun and the genetically determined growth potential. These interactions provide at least part of the explanation as to why there is such a high degree of variation in lifespan among animals maintained on a single fixed dietary regimen in identical environmental conditions.

Implications of long-lasting effects of early dietary and growth behaviour

The fact that 50% of the total variance in lifespan has been explained on the basis of early life dietary and dietary-related parameters, assigns to diet a major determinant function. No two rats are identical in their dietary preferences and growth characteristics; yet the long term interactions can be calculated with sufficient accuracy as to allow a close prediction of the duration of life of individual animals.

All the weight-related data needed in the mathematical model can be obtained for nearly all the rats before 150 d of age. The dietary data can be obtained before they reach 100 d. The quantity of protein consumed and the protein-carbohydrate ratio of the diet selected during days 43-49 are most critical, since substitution of data obtained later greatly reduces the accuracy of the lifespan forecast.

The temporal specificity implies that the self-determined dietary habits and body weight changes during the mature phase have little bearing on lifespan. This assumes that the same mode of feeding is kept throughout postweaning life and that essential constituents needed for life are always available. Additional persuasive evidence of the importance of the early dietary environment comes from fixed diet studies^{10,18,24,27-31}. Certain forms of nutritional intervention limited to early life modify life expectancy and disease risk, whereas dietary intervention later in life may not result in the same degree or type of response. We cannot, however, extend this proposal to include the role of dietary fat^{26,28,32,33} and of the micronutrient^{43,34,35} components, since their relative importance and age relationships were not investigated. Nevertheless, the temporal specificity demonstrated here raises the question whether, through a physiological imprinting mechanism, the variables serve as determinants or signals of an existing underlying programme of events to take place later in life. The data obtained through short term self-selection studies indicating that dietary preference of young animals may have a genetic basis³⁸⁻³⁹, supports the latter possibility. Conversely, the compensatory changes in dietary preference associated with a variety of conditions that alter metabolism⁴⁰⁻⁴⁶ supports the determinant view.

Throughout the greater part of life, the rats were vigorous, clean and free from symptoms of disease. At death, however, a pathological process could be assigned in nearly every case as a contributory or proximate "cause of death"⁷²⁰. Tumours of different tissue origin occurring in many sites and other age-related affections of the lung, heart, kidney, and prostate were particularly prominent among the rats in this study. The ability to make a long range lifespan forecast is therefore all the more remarkable, in that it incorporates differences in the age of onset and the rate of progression of one or more of the debilitating, fatal diseases. It is difficult not to conclude that the individual specificity in early life conditions that govern lifespan are interwoven with those that regulate the susceptibility to several age-related diseases.

The models developed for rats maintained in an unvarying laboratory environment are not directly applicable to other mammals and probably not even for the same stock of rats

living in natural conditions, experiencing many environmental forces. The feeding methods and analytical procedures are applicable, however, to other animals to determine whether the processes that regulate lifespan and disease susceptibility have common denominators and if so, to develop a model that is not restricted to one species. The self-selection method of feeding also allows determination of the extent to which normal physiological or environmentally caused perturbations in appetite and growth at different ages modify length of life and disease risk. At the very least, our observation on the consequences of dietary habits and growth responses early in life have immediate relevance to questions concerned with the appraisal of nutritional adequacy, the relationship between diet and some diseases of age, and the mechanism of the ageing process.

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***gag* Gene of mammalian type-C RNA tumour viruses**

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The translation product of the gag gene of mammalian type-C RNA viruses is a 65,000–68,000 molecular weight precursor polypeptide (Pr65) whose cleavage leads to the formation of four virion proteins, p30, p15, p12 and p10. An immunological approach has been used to establish the arrangement of the sequences coding for these proteins within the viral genome as (5') p15–p12–p30–p10 (3').

TYPE-C RNA viruses have been implicated as causative agents of leukaemias and sarcomas in many vertebrates. A genetic approach to the understanding of type-C virus-coded functions may be useful in determining the mechanisms of replication and transformation by these agents. It is well established that translational products of the viral genome include a reverse transcriptase, envelope glycoprotein, and several lower molecular weight proteins¹, some of which are known to be synthesised as part of a precursor polypeptide^{2–7}. The regions of the viral genome coding for these functions have been termed *pol*, *env*, and *gag* genes, respectively⁸. Another gene, designated *src* (ref. 9), has been identified in transforming type-C viruses of avian and mammalian origin. Evidence obtained by the isolation of conditional and non-conditional mutants of sarcoma viruses indicates that the translation product(s) of the *src* gene are involved in cell transformation (for review, see refs 10–12). Whether type-C viruses, which do not transform fibroblasts in culture but induce lymphoid tumours *in vivo*, contain genes analogous to *src* responsible for lymphoid cell transformation is not yet known.

Among approaches used in attempts to sequence the type-C viral genome, the oligonucleotide fingerprint method has been useful in mapping regions near the poly(A)-containing 3' end of the genome. Analysis of deletion mutants of avian sarcoma viruses by this method has led to mapping of the *env* and *src* genes^{9,13}. Information concerning the composition and partial sequence of the avian *gag* gene has been obtained by techniques involving immunoprecipitation of pulse-labelled polypeptides synthesised by pactamycin-treated cells¹⁴. There is as yet no evidence concerning the genetic sequence of mammalian type-C viruses. There are, however, reports in which immunoprecipitation techniques

have been used to detect a variety of intermediate precursor polypeptides involved in the synthesis of mature virion components^{6,15}.

A different approach to the definition of the composition and sequence of type-C virus-coded genes takes advantage of highly sensitive and specific competition radioimmunoassays for detection and identification of the known structural components of various mammalian type-C viruses. By such procedures, it has recently been shown that one class of temperature-sensitive mutants of Rauscher-murine leukaemia virus (MuLV) is restricted at the non-permissive temperature in cleavage of a precursor polypeptide⁴. We report here an analysis of the size and composition of this viral precursor polypeptide and of its partial cleavage-products. Analogous studies have been performed with cells infected with several naturally-occurring mammalian transforming type-C viruses, that express different numbers of type-C viral structural proteins^{16–18}. The present studies make it possible to establish tentatively the components of the *gag* gene of mammalian type-C viruses, as well as to determine its genetic sequence.

Proteins coded by *gag* gene

Table 1 summarises some of the biochemical and immunological properties of type-C viral proteins for which competition immunoassays were performed. These proteins include gp70 (refs 19, 20), p30 (ref. 22), p15 (ref. 23), p12 (refs 17), and p10 (ref. 24). Each has been demonstrated to be immunologically distinct from the others and to retain its antigenic characteristics when the virus is grown in cells of different species, indicating that each represents a mature translational product of the viral genome. In the first series of experiments, the distribution of these viral proteins was examined in extracts of NIH/3T3 mouse cells infected at the permissive (31 °C) or non-permissive (39 °C) temperature with *ts25*, a Rauscher-MuLV mutant defective in precursor polypeptide cleavage⁴. Post-microsomal cell extracts were subjected to Agarose gel filtration under denaturing conditions (6 M GuHCl). As shown in Fig. 1A, the antigenic reactivities of viral proteins in extracts of cells infected at 31 °C, eluted at molecular weight regions corresponding to those of each of the respective mature proteins isolated from intact virions.

When the same cells were infected at 39 °C instead, antigenic reactivities corresponding to all four proteins, p30,

Table 1 Biochemical and immunological characteristics of type-C viral structural proteins*

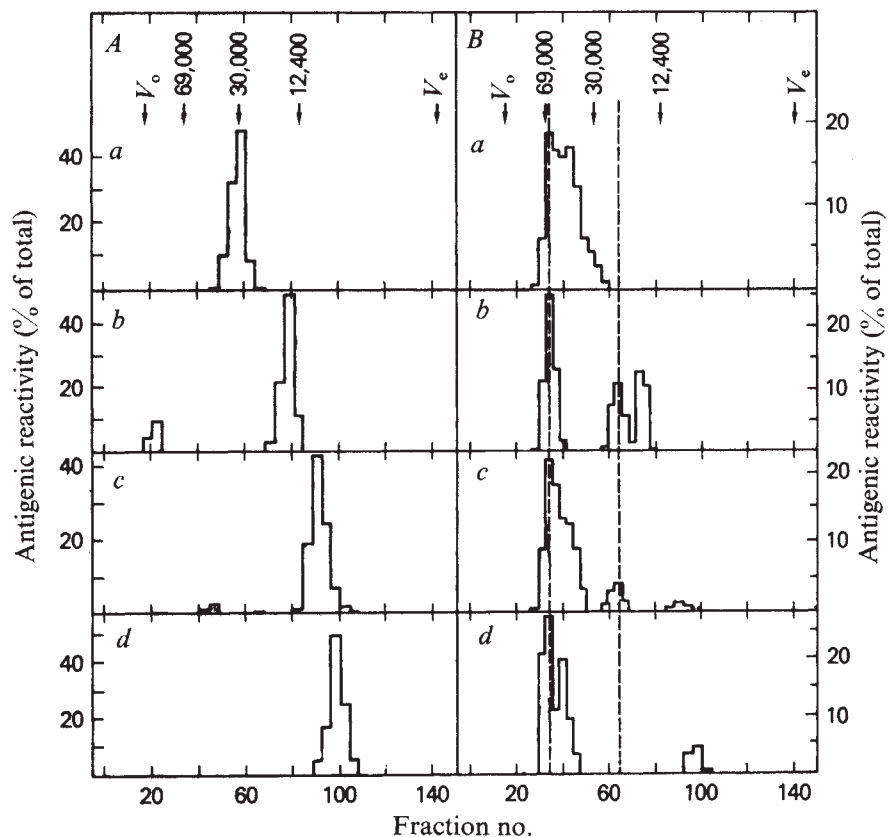
Viral protein†	Molecular weight ($\times 10^{-3}$) (6 M GuHCl)	Phosphocellulose elution (pH 6.5)	Primary antigenic determinants	Other characteristics
gp70	69–71‡	0.1–0.2 M KCl	Type, group, interspecies	Envelope glycoprotein; involved in neutralisation
p30	28–31	0.3–0.4 M KCl	Group, interspecies	Major internal antigen
p15	15–17	0.4–0.5 M KCl	Type, group, interspecies	High tendency to aggregate
p12	11–12	Not bound	Type, group	Phosphoprotein
p10	8–10	0.6–0.8 M KCl	Group, interspecies	Found in ribonucleoprotein complexes

*The data presented in this Table have been summarised from refs 1, 41–43.

†Designated according to convention⁴⁰.

‡Determined by sodium dodecylsulphate–polyacrylamide gel electrophoresis.

Fig. 1 Molecular sizing chromatography of Rauscher-MuLV, *ts25* mutant, grown in NIH/3T3 mouse cells at: *A*, the permissive temperature (31 °C), and, *B*, the restrictive temperature (39 °C). Twenty milligrams of the post-microsomal fraction of *ts25*-infected NIH/3T3 cells were lyophilised and resuspended in a 50 mM Tris-HCl pH 8.0 buffer containing 8 M guanidine-HCl (GuHCl), 1 mM EDTA and 20 mM of freshly prepared dithiothreitol. After 30 min incubation at 37 °C, the sample was applied to an A-5 Agarose (100–200 mesh, BioRad) 1.5 × 90 cm column equilibrated with a 6 M GuHCl solution containing 20 mM dithiothreitol, adjusted to a final pH of 6.5 units. The sample was chromatographed at room temperature at a flow rate of 1.6 ml h⁻¹ with a head pressure of 15 cm. Fractions (0.5 ml) were collected, and exhaustively dialysed at 4 °C for 36 h against a 10 mM Tris-HCl pH 7.8 buffer containing 100 mM NaCl, 0.5 mM EDTA and 0.1% Triton X-100. Samples were tested at twofold serial dilutions in homologous competition radioimmunoassays for Rauscher-MuLV: *a*, p30; *b*, p15; *c*, p12; and *d*, p10 proteins, as described in Table 2. Results are expressed as the percentage of total antigenic reactivity. Molecular weight standards, chromatographed along with the sample, included ¹²⁵I-labelled bovine serum albumin (69,000), ¹²⁵I-labelled Rauscher p30 (30,000), cytochrome *c* (12,400) and ¹²⁵iodine (125). The vertical dotted lines indicate the chromatographic positions of Pr65 and Pr25 precursor polypeptides.



p15, p12, and p10, migrated as a 65,000–68,000 molecular weight precursor polypeptide termed Pr65 (Fig. 1*B*). No reactivity corresponding to any of these proteins was detectable at any higher molecular weight, indicating that if Pr65 is initially synthesised as a precursor polypeptide with a molecular weight greater than 65,000, it must be efficiently cleaved. The size of Pr65 would seem to account for not more than the entire translational information of the four viral proteins coded for by the *gag* gene of Rauscher-MuLV.

The results in Fig. 1*B* also indicate the existence of polypeptides containing p30, p15, p12 and p10 antigenic determinants, and possessing molecular weights less than 65,000. These results suggest that even at the restrictive temperature some precursor cleavage takes place in cultures infected with *ts25* mutant. It was reasoned that systematic analysis of such intermediate cleavage products should provide information concerning the virus maturation process. More importantly, this approach might also provide evidence needed for mapping of individual proteins within the viral genome. For example, the detection of a 23,000–26,000 molecular weight polypeptide containing p15 and p12 antigenic determinants (Fig. 1*Bb* and *c*), suggested that these proteins were located at adjacent positions within the Pr65 precursor polypeptide. Further, antigenic reactivities of p30, p12 and p10 but not of p15 were found in polypeptides eluting in the 40,000 to 60,000 molecular weight region of the guanidine HCl column. In combination with findings of relatively higher levels of mature p15 compared with the other viral proteins, the above data suggested that p15 might be located at a terminal position in the *gag* gene translational product.

Use of mammalian sarcoma virus-transformed cells in sequencing type-C viral genes

Mammalian sarcoma viruses are defective in functions needed for their replication as infectious viruses (for review, see ref. 12). Helper functions including the viral envelope

and reverse transcriptase are conferred by a type-C helper leukaemia virus, invariably present in sarcoma virus stocks. Molecular hybridisation studies have indicated that mammalian sarcoma viruses do contain some helper virus genetic sequences^{25,26}. In fact, several sarcoma viruses express specific helper viral proteins immunologically detectable in transformed cells^{16,18}. That these properties are genetically stable^{18,27,28} provides independent evidence of the association of helper viral sequences with these transforming viral genomes. The viruses analysed here included the S⁺L⁻ (ref. 16) variant of Moloney-murine sarcoma virus (MSV), a natural sarcoma virus isolate from the BALB/c mouse²⁹, designated BALB-MSV, and a sarcoma virus isolated from the woolly monkey³⁰, designated woolly monkey sarcoma virus (WSV). Another virus, the Abelson-murine leukaemia virus (Abelson-MuLV), which induces lymphomas rather than sarcomas³¹, was also examined. Like the S⁺L⁻ Moloney-MSV, this virus transforms fibroblasts in culture and is defective for replication³². The origins and designations of cell lines transformed by each virus are summarised in Table 2.

The specific antigenic determinants of viral proteins expressed by S⁺L⁻ Moloney-MSV and Abelson-MuLV-transformed cells were found to be those of Moloney-MuLV by analysis in radioimmunoassays for type-specific antigenic determinants of p12 and p15 viral proteins, while BALB-MSV transformants expressed antigens specific for a mouse cell tropic helper leukaemia virus indistinguishable from AKR-MuLV by these techniques (data not shown). WSV-transformants contained antigens specific for the woolly monkey helper virus¹⁸. Thus, each transformed cell line and its appropriate uninfected parental line were compared for reactivity in immunoassays for proteins of the specific helper virus in question (Table 2). Uninfected human and rat cells showed no detectable reactivity in any of the assays used (<10 ng of viral antigen per mg cellular protein). Mouse cells that are known to express antigens of their endogenous viruses^{33–35} were, however, cross

Table 2 Patterns of type-C viral antigen expression in cells infected with different mammalian transforming viruses

Transforming virus	Species	Designation	Reference	Levels of viral protein* (ng of antigen per mg cell extract)				
				gp70	p30	p15	p12	p10
NP Moloney-MSV	Mouse	M-NIH	27	<20	<10	<10	<10	<40
	Rat	M-NRK	27	<10	<10	<10	<10	<10
S ⁺ L ⁻ Moloney-MSV	Mouse	D56	16	80†	700	1,700	1,300	<40
	Rat	S ⁺ L ⁻ -NRK	27	<10	800	1,500	1,300	<10
	Human	HuACl ₁	44	NT‡	2,000	4,500	3,900	<10
	Rat	S ⁺ L ⁻ -NRK infected with Moloney-MuLV		3,300	7,600	4,100	3,700	3,400
Abelson leukaemia virus	Mouse	ANN-1	32	<20	<10	1,900	1,100	<40
BALB-MSV	Rat	MAI-3206	Unpublished	<10	<10	600	400	<10
Woolly monkey sarcoma virus	Rat	WSV Cl 9	18	<10	<10	NT	<10	<10
	Rat	WSV Cl 11	18	<10	100	NT	100	100
	Rat	WSV Cl 9 infected with woolly monkey helper virus		6,100	5,100	NT	3,400	3,700

Viral structural proteins of Rauscher-, Moloney- and AKR-MuLV as well as from the woolly monkey helper virus isolate were purified to apparent homogeneity by a combination of ion exchange and molecular sizing chromatographic techniques as described^{20,24}. These included the gp70, p30, p12 and p10 viral proteins of all the four viruses as well as the p15 protein of Rauscher- and Moloney-MuLV. High specific activity ¹²⁵I-labelling of these proteins was achieved by the chloramine T method⁴⁵. After three cycles of freezing and thawing cells were sonicated for 1 min (Biosonic II sonic oscillator) in a 10 mM Tris-HCl pH 7.8 buffer containing 100 mM NaCl, 0.5 mM EDTA and 0.5% Triton X-100, and centrifuged for 2 h at 100,000g. The final supernatant was divided into aliquots and stored at -70 °C. These post-microsomal fractions were tested at twofold serial dilutions in the appropriate competition radioimmunoassays for their ability to displace the ¹²⁵I-labelled viral antigen for binding limiting amounts of goat antiserum raised against homologous virus purified on sucrose gradients. Antiserum and unlabelled competing antigen were incubated at 37 °C for 1 h in a 0.15 ml reaction mixture containing 10 mM Tris-HCl pH 7.8, 10 mM EDTA, 0.4% Triton X-100, 1% bovine serum albumin, 0.1% normal goat serum and 10 mM NaCl (p30, p12 assays) or 300 mM NaCl (gp70, p15 and p10 assays). Around 10,000 c.p.m. of ¹²⁵I-labelled antigen contained in 0.05 ml of the same buffer were added and incubation continued for 3 h at 37 °C and a further 18 h at 4 °C. Following the addition of 0.025 ml of undiluted swine anti-goat immunoglobulin G to each tube to precipitate the antigen-antibody complexes, reaction mixtures were incubated for 1 h at 37 °C and 3 h at 4 °C. Finally, 1 ml of cold 10 mM Tris-HCl pH 7.8 buffer containing 100 mM NaCl and 0.1% Triton X-100 was added to each tube, samples were centrifuged (2,500 r.p.m. for 15 min), supernatants were aspirated, and the radioactivity in the precipitates measured in a Searle gamma counter Model 1285. The results were calculated on the basis of the displacement of the complete competition curve for the unknown relative to that achieved with the corresponding purified viral antigen. Protein concentrations were determined according to Lowry *et al.*⁴⁶ using bovine serum albumin as standard.

*Moloney sarcoma- and Abelson leukaemia virus-transformed cells were tested in homologous immunoassays for Moloney-MuLV proteins. MAI 3206 was assayed in the AKR-MuLV homologous assays except for p15, where anti-AKR-MuLV: ¹²⁵I-Rauscher p15 was utilised. Finally, woolly monkey sarcoma virus-transformed cells were tested in immunoassays for woolly monkey helper virus proteins. Controls for the specificity of each immunoassay included homologous assays for the analogous protein of at least one immunologically distinct type-C virus as well as for the p30s of avian myeloblastosis and RD114 viruses.

†Slightly raised levels of gp70 were observed only in S⁺L⁻ Moloney-MSV-transformed mouse cells. This gp70 was, however, found to contain type-specific antigenic determinants of an endogenous mouse type-C virus⁴⁷ (data not shown). These findings together with the lack of detectable gp70 in S⁺L⁻ NRK rat cells indicate that this gp70 is not coded for by the S⁺L⁻ Moloney-MSV genome.

‡N.T., Not tested.

reactive at low levels (<50 ng of viral antigen per mg cellular protein) in assays for Moloney-MuLV gp70 and p10 proteins. Therefore, these were subtracted from values obtained with each virus-transformed mouse cell line. As a positive control, virus-transformed cells productively infected with the appropriate helper virus produced high levels of each of the five viral antigens tested.

As shown in Table 2, none of the transformants analysed produced significant amounts of the envelope glycoprotein, gp70. In the analysis of *gag* gene products, several different patterns of expression were observed. For example, cells transformed by the non-producer (NP) variant of Moloney-MSV and by one genetically stable variant of WSV (WSV Cl 9), demonstrated no antigen expression above the levels observed in uninfected parental cells. In contrast, mouse, rat or human cells transformed by the S⁺L⁻ variant of Moloney-MSV expressed high levels of three Moloney-MuLV proteins, p30, p15 and p12. The production of the same antigens in cells of different species strongly indicates that they are coded by the virus. The fact that p10 was the only missing protein among the Moloney-MuLV *gag* gene products expressed by S⁺L⁻ Moloney-MSV-transformed cells suggested that this low molecular weight protein might be located at the carboxy-terminal end of the *gag* gene precursor polypeptide.

BALB-MSV and Abelson-MuLV transformants demonstrated high levels of p15 and p12 viral proteins in the absence of p30 or p10, indicating that p30 is the viral protein in closest proximity to p10. These results do not establish the positions of p15 and p12 viral proteins with respect to each other. The presence of *ts25* Rauscher-

MuLV-infected cells of a 23,000–26,000 molecular weight intermediate cleavage product containing both antigens, along with the results presented in Table 2, suggests, however, that p15 and p12 occupy adjacent positions at the amino-terminal region of the *gag* gene translational product.

Transformants of one WSV variant (WSV Cl 11) expressed readily detectable levels of p10 in addition to p30 and p12 woolly monkey helper virus proteins. The unavailability of a radioimmunoassay for woolly monkey viral p15 made it difficult to establish directly the presence of this antigen. The cells were, however, found to express a 65,000–68,000 molecular weight polypeptide containing p30, p12 and p10 and another of 23,000–26,000 molecular weight, possessing p12 but not p30 or p10 (data not shown). Their sizes are evidence that these polypeptides are analogous to those found in Rauscher-MuLV-infected cells and, therefore that p15 is present in both polypeptides. Thus, the WSV Cl 11 transforming virus seems to contain sequences accounting for the entire helper viral *gag* gene.

p15 is the amino-terminal protein of the *gag* gene translational product

In an attempt to determine the relative position of p15 and p12 within the *gag* gene Pr65 product, the molecular size distribution of individual viral antigens was examined in cell extracts of certain of the mouse virus-transformed cell lines described above. The results presented in Figs 2–4 demonstrate the existence of high molecular weight precursor polypeptides as well as several intermediate cleavage products containing *gag* gene-coded structural proteins. Each transformant exhibited a 23,000–

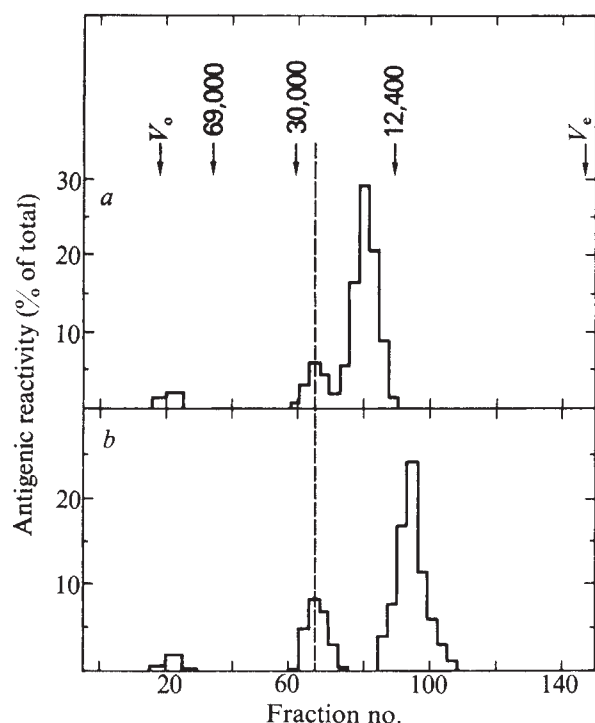


Fig. 2 Molecular sizing chromatographic analysis of helper leukaemia virus-specific antigens expressed in BALB-MSV-transformed rat cells. Twenty-five milligrams of a post-microsomal fraction were submitted to gel filtration chromatography under denaturing conditions as described in the legend to Fig. 1. Dialysed fractions were tested at twofold serial dilutions in the following competition radioimmunoassays: *a*, anti-AKR-MuLV ^{125}I -Rauscher-MuLV p15 assay; and *b*, anti-AKR-MuLV: ^{125}I -AKR-MuLV p12 assay. Results are expressed as percentages of total antigenic reactivity. Molecular weight standards are those described in the legend to Fig. 1. The vertical dotted line represents the chromatographic position of the Pr25 precursor polypeptide.

26,000 molecular weight precursor polypeptide (Pr25) containing p15 and p12 viral proteins analogous to the intermediate cleavage product of the Rauscher-MuLV *ts*25 mutant (Fig. 1*B b* and *c*). The identification of this polypeptide as a precursor to p15 and p12 was particularly obvious in BALB-MSV (Fig. 2) and Abelson-MuLV (data not shown) transformed cells that lacked either p30 or p10. These findings indicate that sequences coding for p15 and p12 occupy adjacent positions within the viral genome.

Analysis of S^+L^- Moloney-MSV-transformed mouse cells revealed the presence of additional intermediate cleavage products involved in the synthesis of viral antigens (Fig. 3). These included a 58,000–62,000 molecular weight precursor polypeptide (Pr60) containing p30, p15, and p12 but not p10 and a 43,000–47,000 molecular weight precursor polypeptide (Pr45) containing p30 and p12 antigens in the absence of p15 or p10. The finding that Pr60 lacks only p10 provides further evidence that this low molecular weight protein occupies the carboxy-terminal position in the complete *gag* gene-coded precursor polypeptide. Moreover, the detection of a precursor containing p30 and p12 (Pr 45), but lacking p15, argues for the adjacent location of p30 and p12 within the *gag* gene product. As shown in Fig. 4, the results obtained with S^+L^- Moloney-MSV-transformed human cells provide additional evidence supporting this possibility. Cell extracts of this transformant contained proportionally less Pr60 but demonstrated considerably increased amounts of the Pr45 intermediate cleavage product containing p30 and p12, but not p15.

The identification of the intermediate cleavage products, Pr25 and Pr45, led to the establishment of two pairs of

adjacent proteins, p15–p12 and p12–p30, respectively. These findings imply that p12 is located between p15 and p30. Moreover, since p30 is missing in BALB-MSV- and Abelson-MuLV-transformed cells that express p15 and p12, the location of p15 must be at the amino terminal end of the *gag* gene translational product.

Sequence and tentative mapping of the *gag* gene

The present evidence utilising a Rauscher-MuLV *ts* mutant blocked in precursor polypeptide cleavage indicates that the *gag* gene translational product (Pr65) contains the major internal protein, p30, as well as the low molecular weight proteins, p15, p12, and p10 (Fig. 1). Moreover, analysis of the expression of helper virus-coded proteins in cells infected with replication-defective transforming viruses revealed that p10 is the carboxy-terminal protein of the *gag* gene-coded precursor polypeptide, and that p30 is adjacent to it (Table 2). Analysis of intermediate cleavage products of the Pr60 polypeptide provided information necessary for the relative positioning of p15 and p12 (Figs 3 and 4). These results led to the following sequence of the *gag* gene product: $\text{NH}_2\text{-p15-p12-p30-p10-COOH}$. Since the viral RNA genome is the positive strand³⁶, the genetic information contained in the *gag* gene would then be arranged in an identical manner from the 5' to 3' end of the RNA molecule. A summary of these findings is presented in Fig. 5.

The sequence of the mammalian type-C viral *gag* gene was deduced from analysis of a conditional lethal mutant of mouse leukaemia virus defective in precursor polypeptide

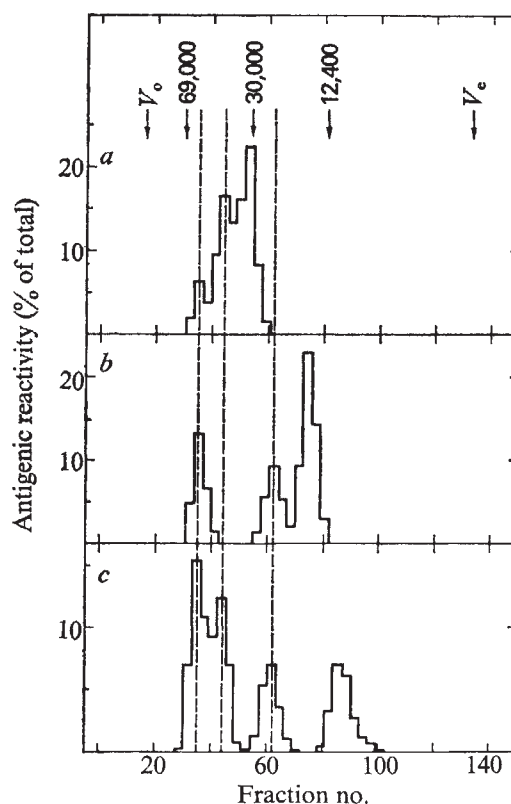


Fig. 3 Molecular sizing chromatographic analysis of helper leukaemia virus-specific antigens expressed in S^+L^- Moloney-MSV transformed mouse cells. Twenty mg of a post-microsomal fraction were submitted to gel filtration chromatography under denaturing conditions as described in the legend to Fig. 1. Samples were tested in homologous competition radioimmunoassays for Moloney-MuLV: *a*, p30; *b*, p15; *c*, p12; proteins as described in Table 2. Results are expressed as the percentage of total antigenic reactivity. Molecular weight standards are those described in Fig. 1. The vertical dotted line indicates the elution positions of Pr60, Pr45, and Pr25 *gag* gene-coded precursor polypeptides.

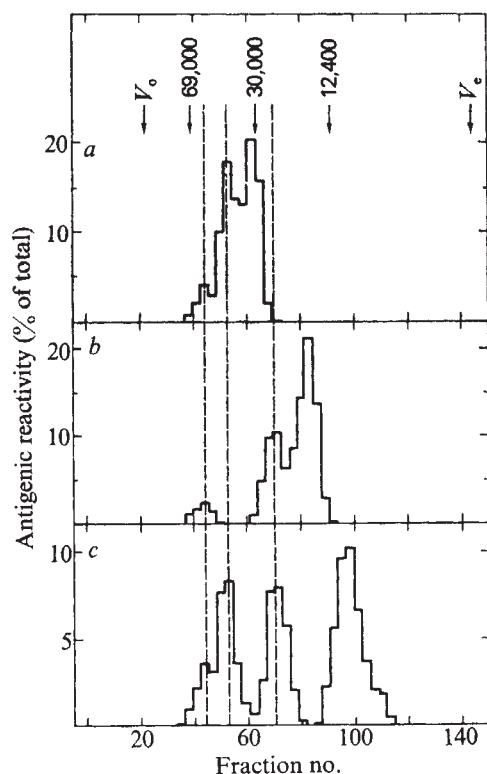
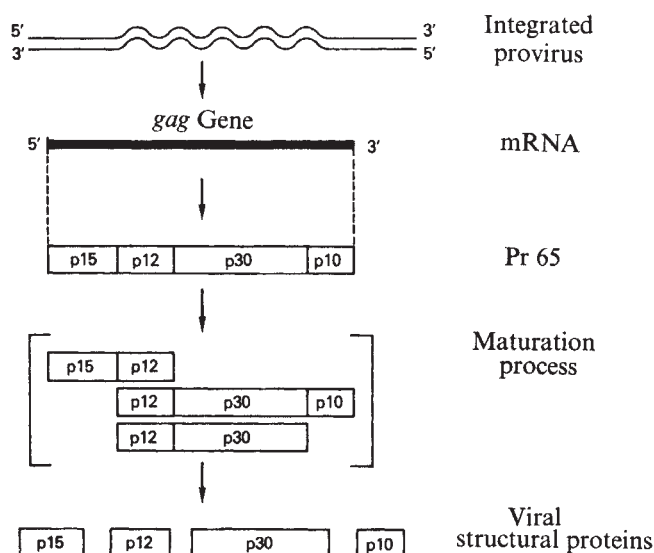


Fig. 4 Molecular sizing chromatographic analysis of S^+L^- Moloney-MSV-transformed human cells. The analysis of Moloney-MuLV helper antigens was performed as indicated in Fig. 3. Vertical dotted lines represent the elution positions of Pr60, Pr45 and Pr25 gag gene-coded precursor polypeptides.

cleavage and several independent isolates of replication-defective transforming viruses. These viruses contain stably-associated genetic information of their respective helper leukaemia viruses. Specific patterns of expression of gag gene helper viral proteins were demonstrated in cells infected with these transforming viruses. In each case, the size of the highest molecular weight precursor polypeptide corresponded to that expected for a helper leukaemia viral precursor containing only those gag gene translational products. Furthermore, the intermediate cleavage products detected in cells infected with each transforming virus

Fig. 5 The sequence and maturation process of the gag gene translational product of mammalian type-C RNA viruses.



were analogous to those detected in cells infected with the conditional lethal mutant of MuLV. The final cleavage products were also identical in sizes and immunological properties to the mature viral proteins of the helper leukaemia virus. These findings all argue against any drastic rearrangement of gag gene sequences in sarcoma as opposed to helper leukaemia viruses.

Our studies of several independent isolates of transforming viruses were consistent with only one possible ordering of gag gene sequences on the basis of the above reasoning. Thus, if this gene order were to result from some alteration of the gag gene normally present in the helper leukaemia virus, this alteration must be highly specific. The ability of different genetic variants of the same transforming virus to transform cells does not seem to be affected by whether they express particular gag gene products^{18,27}. Thus, the possibility of a specific genetic rearrangement of gag gene sequences having any functional significance in the transforming virus seems highly unlikely. Instead, the evidence strongly argues that the gag gene sequence as deduced from studies of defective mammalian transforming viruses represents the gag gene sequence of the helper leukaemia virus.

With avian type-C viruses, a different experimental approach has led to the following partial sequence of the avian gag gene 76,000 molecular weight translational product: NH_2 -p19-(p12-p27)-p15-COOH (ref. 14). The position of p12 with respect to p27 is not resolved. Moreover, another low molecular weight protein, p10 (ref. 37) has not been identified within this precursor. Mapping studies of gag gene may make it possible to assign similar functions to viral proteins of avian and mammalian type-C viruses on the basis of their positions within their respective viral RNA genomes.

Genetic mapping of env and src genes at the 3' end of the avian sarcoma viral genome^{9,15} leaves only the gag and pol genes among the known viral genes that may exist at the 5' end of the genome. The present evidence does not make it possible to assign the position of the gag gene within the mammalian type-C viral RNA molecule. When helper leukaemia viral translational products were detected in the virus transformed cells studied here, however, they invariably included gag gene products in the absence of detectable glycoprotein (Table 2). Moreover, one cell line tested, a S^+L^- Moloney MSV transformant of human origin, has been shown to lack detectable viral reverse transcriptase as well⁴. These findings, together with recent reports concerning *in vitro* synthesis of gag gene products directed by 35S viral RNA^{3,7} indicate that an initiation codon must exist at (or close to) the 5' end of the gag gene. Thus, if the viral genome were to contain a single protein synthesis initiation site, the above information would be sufficient to map the gag gene at the 5' end of the viral genome. Reports concerning the size of the viral mRNA^{38,39} as well as its translational products⁸, taken together would argue for a single initiation site. This possibility remains speculative, however, and awaits further experimental verification.

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letters to nature

Solar neutrinos and solar accretion of interstellar matter

MCCREA¹ has recalled the suggestions^{2,3} that the gravitational energy released by material accreted by the Sun from interstellar clouds could trigger the onset of terrestrial Ice Ages. The purpose of this communication is to point out that if the Hoyle–Lyttleton mass accretion rate applies, this accretion is sufficient to enhance the surface heavy element abundances of the Sun and other solar-type stars. The enhancement may be sufficient to allow the construction of consistent solar models with an interior heavy element abundance significantly lower than the observed surface abundance. This condition lowers the predicted solar neutrino flux⁴. Joss⁵ has suggested that a similar enhancement of surface abundances might occur by accretion of planetesimals left over after formation of the Solar System. Both mechanisms may occur, thereby increasing the effect.

In the simple accretion model of Hoyle and Lyttleton³ a body of mass M accretes matter at a rate

$$M = 2.4 \times 10^{22} (M/M_{\odot})^2 n(\text{H}_2) V^{-3} \text{ g yr}^{-1} \quad (1)$$

where $n(\text{H}_2)$ is the equivalent number of hydrogen molecules cm^{-3} and V is the velocity relative to the cloud in km s^{-1} .

If the clouds with internal densities of $n_c(\text{H}_2)$ are distributed randomly with N_c clouds pc^{-3} , and if they may be characterised by spheres of radius, R_c pc, then the fraction of the volume of the galactic disk occupied by cloud material will be $f_c = N_c(4\pi/3)R_c^3$. For the age, A , of the Sun, the fraction of time spent inside such clouds will be f_c , and consequently the total mass accreted will be

$$\delta M \simeq \langle M f_c \rangle A = 8.2 \times 10^{-2} M_{\odot} \langle f_c n_c(\text{H}_2) V^{-3} \rangle \quad (2)$$

The product $f_c n_c(\text{H}_2)$ is the density of H_2 which would result from smoothing out clouds into a uniform density disk. It is important to note that to compute δM one should include only those clouds for which the internal cloud densities are large enough to choke off the solar wind flow and allow material to accrete on to the stellar surface. As pointed out below, this is approximately $n_c(\text{H}_2) = 10^4 \text{ cm}^{-3}$. From Gordon and Burton⁶ we adopt the smoothed out density at 10 kpc from the galactic centre of $\langle n(\text{H}_2) \rangle = f_c n_c(\text{H}_2) = 0.5 \text{ cm}^{-3}$. This figure is based

on H_2 deduced from CO molecular lines, which require $n_c(\text{H}_2) \gtrsim 10^4 \text{ cm}^{-3}$, and consequently it includes the type of clouds which will cause accretion. Consequently, we obtain

$$\delta M \simeq 4 \times 10^{-2} M_{\odot} \langle V^{-3} \rangle \quad (3)$$

Interstellar H I clouds have a turbulent velocity dispersion of $\sim 6.4 \text{ km s}^{-1}$ (ref. 7), the mean gas velocity changes by several km s^{-1} during the full cycle of a galactic spiral density wave⁸, and the velocity of the Sun oscillates $\sim 20 \text{ km s}^{-1}$ around its mean motion about the galactic centre. Thus one expects the relative velocity of the Sun and interstellar clouds to vary over the range $0 \sim 30 \text{ km s}^{-1}$. For any reasonable distribution function of cloud velocities $\langle V^{-3} \rangle$ diverges; consequently the figure to use in equation (3) is dominated by rare, low velocity encounters.

To illustrate this sensitivity, consider the 37 clouds studied by Hobbs⁹. The velocity dispersion of the clouds relative to the Sun was $\langle V^2 \rangle^{1/2} = 15.7 \text{ km s}^{-1}$ (note that this figure includes the systematic motions as well as what would be called turbulent motions about the mean cloud motions observed towards each star). In contrast, the quantity appropriate for use in equation (3) is $\langle V^{-3} \rangle^{-1/3} = 3.1 \text{ km s}^{-1}$. To emphasise the sensitivity to rare encounters of low relative velocity, if the one cloud towards ϵ Aur with velocity 1 km s^{-1} is dropped from the mean, then $\langle V^{-3} \rangle^{-1/3} = 5.4 \text{ km s}^{-1}$.

It is not clear whether this sample of clouds is broad enough to determine the appropriate figure to use in equation (3), especially since these clouds are not the same type of objects which have densities of $n_c(\text{H}_2) \gtrsim 10^4 \text{ cm}^{-3}$. Nevertheless, we expect that the appropriate mean velocity is likely to be in the range $2\text{--}5 \text{ km s}^{-1}$ and this gives

$$\delta M \simeq 5 \times 10^{-3} \text{ to } 3 \times 10^{-4} M_{\odot} \quad (4)$$

A crucial question which must be answered by future research is whether or not accretion on to the solar surface will actually occur. One of the most obvious obstacles is the outward flowing solar wind. A simple computation may be done by setting the dynamic pressure of the solar wind flow equal to the dynamic pressure of the dense interstellar gas¹⁰. It is easy to show that for interstellar cloud densities of $n(\text{H}_2) \simeq 10^3 \text{ cm}^{-3}$ the solar wind terminates at $\sim 1 \text{ AU}$. For larger densities the heliosphere

is compressed further and for densities of a few 10^3 cm^{-3} the termination is within a few solar radii of the Sun. To decide what occurs for higher densities requires knowledge of the mechanisms which affect the solar wind within its critical sonic point at a few solar radii. It appears that the outward flow can be reversed to an inward flow for cloud densities which characterise the dense H_2 clouds used for the estimate in equation (5).

If the accreted mass contains a heavy element abundance by mass $Z_g(t)$ and it has been continually mixed into a surface convection zone of mass ΔM , then the surface abundance of heavy elements will be

$$Z_s(t) = e^{-y(t)} \left[Z_0 + \int_{t_0}^t e^{y(t')} Z_g(t') \frac{\dot{M} dt'}{\Delta M} \right] \quad (5)$$

where Z_0 is the heavy element abundance of the star at its time of formation t_0 , and

$$y(t) = \int_{t_0}^t \frac{\dot{M} dt'}{\Delta M}$$

The presently observed solar Z_s is $\sim 1.5 \times 10^{-2}$. The heavy element abundance of the interstellar gas at the present time, T , deduced from young stars and H II regions is about the same. If the heavy element abundance in the gas has increased with time and therefore $Z_g(t) > Z_0$, then equation (5) is satisfied providing $y(T) \gtrsim 1$. Standard solar models give surface convection zones with $\Delta M \simeq 10^{-2} M_\odot$. This together with the predicted range for δM given in equation (4) seems to indicate $y(T)$ somewhat < 1 . Ulrich (personal communication) has, however, shown that ΔM is even smaller if the interior abundance Z_0 is smaller than the surface abundance Z_s . Therefore for sufficiently low values of Z_0 it seems that equation (5) will be satisfied.

The initial solar value Z_0 is difficult to estimate. It is most likely substantially greater than zero. Simple models for the evolution of the Galaxy (for example ref. 11) suggest that 4.5×10^9 yr ago the interstellar gas may have had a value of Z at least as great as $\frac{1}{3}$ – $\frac{2}{3}$ the present value Z_g . If these values for Z_0 reduce ΔM to approximately the size of δM , then the solar neutrino counting rate is reduced by a factor of $\sim (Z_0/Z_s)^{1.1}$ (ref. 12) to 2.6–3.6 SNU.

Although the precise magnitude of the effect is still uncertain, it seems probable that some enhancement of the solar surface heavy element abundance must occur. Hence, it appears that this mechanism could significantly reduce the discrepancy between the observations and predictions of solar models.

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Solar neutrinos and galactic contamination of the Sun

ONLY recently-published observations permit a useful estimate of the total accretion of interstellar matter (ISM) by the Sun since it became a main sequence star. Using measurements from the OAO-2 satellite, Jenkins and Savage¹ conclude that "... possibly the most secure estimate of the amount of hydrogen within 1 kpc of the Sun is ... an overall density n_H (total) = 1.5 atoms cm^{-3} (in the form of protons, atoms and molecules)". Using this number we suggest here a possible explanation of the low observed solar neutrino rate.

Taking hydrogen to be 70% by mass gives for the density of ISM in units of the atomic mass of hydrogen

$$n \simeq 2 \text{ cm}^{-3} \quad (1)$$

If the Sun of mass 2×10^{33} g traverses ISM of density n at speed V km s^{-1} the mass $m(L)$ accreted in travelling L pc is

$$m(L) = 1.2 \times 10^{28} L n V^{-4} \text{ g} \quad (2)$$

and the total mass $m(T)$ accreted in time $1T$ (in units of 10^{10} yr) is

$$m(T) = 1.2 \times 10^{32} T \langle n \rangle \langle V^{-3} \rangle \text{ g} \quad (3)$$

assuming n , V to be uncorrelated^{2,3}. Now a region of radius 1 kpc around the Sun seems to be quite typical along the path of the Sun in present conditions in the Galaxy. Also the amount of ISM was presumably somewhat greater in the past. So from equation (1) we infer that it is not an overestimate to use in equation (3)

$$\langle n \rangle \simeq 2 \quad (4)$$

If the Sun is in a cloud of ISM the relative speed V is usually taken to be in the range 5–25 km s^{-1} . In equation (3) smaller values are weighted more strongly, so we take

$$\langle V^{-3} \rangle \simeq 10^{-3} \quad (5)$$

According to standard estimates, the Sun reached the main sequence about 5×10^9 yr ago. The total mass $m(T = 0.5)$ accreted since then is estimated from equations (3), (4) and (5) as

$$m \simeq 10^{29} \text{ g} \quad (6)$$

This is an underestimate because it takes no account of the densest parts of the ISM that produce so much obscuration that no stars can be seen through them. Indeed, if the Sun traverses even a single 'very dense' cloud it can accrete between 10^{29} and 10^{31} g, as follows from equation (2) using appropriate values of L , n , V . The Sun must have had a good chance of accreting some such amount each of the ~ 50 times it has crossed a dust-lane associated with a spiral arm of the Galaxy. From all this we may safely take m to be in the range

$$10^{29} < m < 10^{30} \text{ g} \quad (7)$$

and we should not exclude still higher values.

On current models, the Sun has an outer convective zone that, along with a region of convective overshooting immediately beneath it comprising altogether a mass M_{conv} , is itself kept mixed but undergoes no mixing with material

from the interior. Quoting R. K. Ulrich, Joss⁴ gives for two models

$$Z_0 \lesssim 0.002 \quad M_{\text{conv}} \simeq 10^{29} \text{ g} \quad (8)$$

$$Z_0 \simeq 0.02 \quad M_{\text{conv}} \simeq 5 \times 10^{31} \text{ g} \quad (9)$$

Z_0 is the initial heavy-element abundance by mass, here assumed to have the same value throughout a model.

Amongst general conclusions from the literature⁵ on the chemical composition of the stars are: (a) Clouds from which stars are being formed at the present time have Z -values on average 1.5–2 times higher than those from which stars were formed when the Sun was born. (b) There is an intrinsic spread in the Z_0 -values of stars born about any particular time; this is seen especially in the work of Clegg and Bell⁶ who find, for stars younger than the Sun, a sixfold range of Z_0 . At present IMS has $\langle Z \rangle \simeq 0.02$. Allowing a factor 2 on account of (a) and 2 either way on account of (b) would give a range

$$0.005 \lesssim Z_0 \lesssim 0.02$$

for the Sun at birth, and would not exaggerate the possible range.

These bounds for Z_0 give two possible interpretations of the fact that the observed spectrum of the Sun gives $Z \simeq 0.02$:

(A) $Z_0 \simeq 0.005$. Here M_{conv} is near the low value in model (8) and this can be exceeded by m in condition (7). In that case the convective zone is composed of accreted matter for which $Z \simeq 0.02$.

(B) $Z_0 \simeq 0.02$. Here M_{conv} having the high value in model (9) is unlikely to be significantly affected by accretion, and accretion of ISM having $Z \simeq 0.02$ would not in any case affect the composition.

Case (B) is the usual standard uniform model. But from the present standpoint, apart from accretion, a uniform model with $Z_0 \simeq 0.005$ is about as likely as one with $Z_0 \simeq 0.02$; accretion would, however, cause (A) to show the same spectrum as (B). There could be intermediate cases, but we are concerned to demonstrate the plausibility of quite widely different cases.

Bahcall *et al.*⁷ predict for uniform solar models

$$Z_0 = 0.002, \text{ neutrino detection rate } \simeq 1.4 \text{ SNU} \quad (10)$$

$$Z_0 = 0.02, \text{ neutrino detection rate } \simeq 5.6 \text{ SNU} \quad (11)$$

A roughly linear dependence of detection rate upon Z_0 near $Z_0 = 0.015$ inferred earlier⁸ would have required it to go from 5.6 to 1.4 SNU if Z_0 goes from $Z_0 = 0.02$ to $Z_0 = 0.005$. In view of result (10) this may not be quite correct, but it shows the predicted rate to be not much above 1.4 SNU if $Z_0 = 0.05$, that is, for case (A). The result (11) predicts 5.6 SNU in case (B).

As their most up-to-date determination, R. Davis, Jr, and J. C. Evans (private communication) find 1.5 SNU as the 1σ upper bound on the solar neutrino detection rate (see also ref. 9). We suggest as the simplest explanation of this low value the possibility that the Sun actually has a low heavy-element content, except in its convection zone which has been enriched by accretion.

The phenomenon of a considerable difference between surface and interior Z -values because of accretion is likely only for stars not much different from the Sun. Population II stars will have space velocities that are too high for significant accretion to occur. Stars of smaller mass would have deeper convective zones, while stars of much greater mass would generate H II regions tending to inhibit accretion, and would not be old enough for significant changes in Z to develop in the ISM from the time of formation. Therefore our suggestion would not require any changes in composition (Z) for the ISM

from that normally assumed, either for the present period or when the population II stars were being formed.

In a highly suggestive paper, Joss⁴ has considered the possible enhancement of the heavy-element abundances in stellar surfaces by the accretion of chemically fractionated matter in the form of comets and planetesimals. It follows fairly conclusively from his discussion, however, that such material in the required quantity is not available in the case of the Sun. Our suggestion differs from his in a simple way, but it is the crucial one that we appeal only to ISM known to exist in the amount needed.

Amongst possible objections is, for instance, any reason for the inapplicability of the simple theory of accretion; in particular the solar wind might combat accretion, though this seems unlikely to be important since heavy accretion would occur mainly in relatively short intense bursts. Anyhow, little is known about the cause of the solar wind or its time dependence. Again there is the question as to whether the mere addition of material of relatively high Z -value might automatically increase the extent of the convective zone, thereby rendering our process self-defeating. There are also known problems regarding particular abundances (lithium in the Sun, planetary compositions, to name but two) that are still unresolved in any theory. On the other hand, were the neutrino observations accepted as demanding a low Z -value inside the Sun, this would clarify these other problems.

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Climatic effects during passage of the Solar System through interstellar clouds

It seems likely that the Solar System passes through regions where there are a large number of dense interstellar clouds as the compression zone of the galactic spiral density wave passes the Sun. When this occurs, several processes may cause significant changes in the climate of the Earth and other planets. We discuss here the influences of compression of the solar wind cavity, accretion by the Sun, and particulate input into the Earth's atmosphere. The first two may produce radiation hazards for life in addition to causing climate stress.

Shapely¹ and Hoyle and Lyttleton² have discussed the idea that gravitational energy released by the accretion of interstellar material to the Sun at a rate $\dot{M}$ would enhance the solar luminosity. The Hoyle–Lyttleton mechanism gives an enhancement of $\delta L \approx M (GM_{\odot}/R_{\odot}) \propto n(\text{H}_2)/V^3$, where $n(\text{H}_2)$ is the equivalent number of hydrogen molecules per cm^3 and V is the velocity of the Sun with respect to the interstellar medium. In contrast to the small accretion rates predicted from earlier data, McCrea³ has pointed out that there exist very dense interstellar clouds with $n(\text{H}_2)$ values as large as 10^5 – 10^7 cm^{-3} , which may produce $\delta L \approx 10^{-2}(n(\text{H}_2)/10^5 \text{ cm}^{-3})L_{\odot}$ using $V = 20 \text{ km s}^{-1}$. Simple arguments about terrestrial heat balance suggest that

luminosity enhancements of 1% or greater will produce significant variations in climate.

The spiral pattern in the Galaxy sweeps by the Sun at intervals of about 10^8 yr, and the sharp compression behind a shock front is believed to initiate the collapse of some interstellar clouds and to induce star formation; see, for example, Shu *et al.*⁴. The width of the compression zone corresponds to an interval of about 10^6 yr. McCrea has estimated that within each compression zone the Sun would encounter several dense clouds, and that the traversal of each would last about 5×10^4 yr (McCrea also considered how these varied times may relate to observed time scales for ice ages).

Observational data are sparse, but it seems that most lines of sight from the Sun encounter cloud complexes of CO molecules^{5,6} with emission line strengths implying $n(\text{H}_2) \gtrsim 10^3 \text{ cm}^{-3}$. Observed clouds probably have lifetimes of about 10^6 yr, so Earth is unlikely to pass through any of those. If the pattern is continuously maintained by cloud compression in the spiral shock, however, then approximately the same number of clouds can be expected in the compression zone when it passes the Sun. This reinforces McCrea's suggestion³ that the Sun will regularly encounter dense clouds, but the number of such clouds and the probability of encountering very dense clouds with $n(\text{H}_2) \gtrsim 10^5 \text{ cm}^{-3}$ is still uncertain. It seems, however, that encounters with clouds of $n(\text{H}_2) \gtrsim 10^3 \text{ cm}^{-3}$ are almost inevitable. We have analyses (unpublished) suggesting that during its lifetime the Solar System has passed through at least 45 clouds with $n(\text{H}_2) \gtrsim 10^2 \text{ cm}^{-3}$ and at least five clouds with $n(\text{H}_2) \gtrsim 10^3 \text{ cm}^{-3}$.

Dennison and Mansfield⁷ have discussed some of the observational difficulties of McCrea's suggestion that each individual glaciation is caused by passage through a separate cloud (there is apparently no trace of the cloud which would have caused the last glaciation). Those arguments, however, do not vitiate the general idea that passage through interstellar clouds may influence climate.

When the Solar System moves through a cloud, the dynamic ram pressure will balance the outward flowing solar wind at a stand-off distance R_s on the upstream side. Under most circumstances of interest V will be much greater than the sonic speed of the interstellar gas, so the flow will be supersonic on both sides of R_s , the two flows being separated by a relatively thin subsonic region. The position of R_s may be estimated by balancing the dynamic pressures of the two opposing flows. The solar wind dynamic pressure is approximately $p_E = m_H n_E (R_E/R)^2 V_E^2$, where m_H is the mass of the hydrogen atom, $n_E \approx 7 \text{ cm}^{-3}$ is the average density currently at $R_E = 1 \text{ AU}$, and $V_E \approx 400 \text{ km s}^{-1}$ is the velocity at 1 AU. The dynamic pressure of the interstellar gas after falling into R is approximately given by

$$p_G = 2m_H n(\text{H}_2) \left(V^2 + \frac{2GM_\odot}{R} \right) \quad (1)$$

If R is greater than the 'accretion radius' $r_c = GM_\odot/V^2$, then $n(\text{H}_2)$ remains approximately equal to its value at large distances from the Sun, and therefore $p_G \approx \text{constant}$. If $R < r_c$, however, the inflowing gas density increases roughly proportionally to $R^{-3/2}$, and $p_G \propto R^{-5/2}$. With that behaviour the stand-off radius is approximately

$$R_s \approx 750 n(\text{H}_2)^{-1/2} V^{-1} \text{ AU} \quad (2)$$

for small $n(\text{H}_2)$, where V is expressed in km s^{-1} . If $n(\text{H}_2)$ is greater than a critical value of approximately $0.4 V^2 \text{ cm}^{-3}$ pressure balance is no longer possible and the interstellar gas overwhelms the solar wind. When that occurs $R_s \approx 1.2 \times 10^3 V^{-2} \text{ AU}$. That is altered only slightly if non-fluid behaviour resulting from the large mean free path of the neutral gas is considered.

As a typical case we here adopt $V = 20 \text{ km s}^{-1}$ for the velocity of the Sun relative to clouds. For that value of V the critical values of $n(\text{H}_2)$ and R_s are about 160 cm^{-3} and 3.0 AU .

In this analysis the conditions on the downstream side are not clearly discernible; nevertheless it is clear that for clouds of $n(\text{H}_2) > 10^3 \text{ cm}^{-3}$ the Earth will be outside the solar wind cavity for at least part of the year. Furthermore, the flow becomes an accretion flow which may produce a significant alteration of the chemical abundances at the solar surface and, by implication, surface abundances of other stars as well (R.J.T. and M.J.N., unpublished).

Observational evidence suggests that there is some mechanism producing a relationship between solar wind flow and climate⁸⁻¹¹. One proposed mechanism is that contemporary solar wind modulation of galactic cosmic rays influences climate¹²⁻¹⁴. Consequently, the fact that the Earth would be outside the solar wind cavity for all or part of the year may have an effect on terrestrial climate. Outside the heliosphere the Earth will presumably be exposed to the full galactic cosmic ray flux; furthermore, there may be an enhanced flux in those environments because of the supernova deaths of massive stars which spend their entire lives in the vicinity of the dense interstellar clouds from which they form. Ruderman¹⁵ has discussed the consequences of nearby supernovae explosions for atmospheric ozone and terrestrial life.

One discussion about the relationship between solar wind and weather has focused on the passage of the Earth through sector boundaries of the interplanetary magnetic field⁸. If those magnetic field discontinuities are important, then discontinuities encountered as the Earth moves in and out of the heliosphere should also be important.

McCrea's discussion of the accretion luminosity δL considered only its magnitude. Its spectrum will be largely determined by the temperature of the gas after the bow shock at R_s or the accretion shock at the solar surface. The temperature will reach at least 40,000 K, which is the value the gas would attain by converting the energy of 20 km s^{-1} bulk relative motion into thermal energy. The spectral character of this radiation will be quite different from the normal solar spectrum, for it will be mostly ultraviolet and X-ray radiation.

During quiet solar periods, solar radiation contains about $1.5 \times 10^{-6} L_\odot$ on the short wavelength side of Lyman α (ref. 16); this portion governs the thermosphere of the Earth. Therefore, $\delta L \approx 10^{-6} L_\odot$ will produce changes of order unity both in the properties of the Earth's atmosphere above about 150 km and in the ionospheric regions of all planets. Solar radiation contains about $1.5 \times 10^{-2} L_\odot$ between 2,100 and 3,100 Å (ref. 16), the wavelength region influencing the stratosphere.

The suspected relationships between observed, contemporary solar activity and climatic change^{12,13,17} suggest that relatively small variations in solar ultraviolet radiation input may have perceptible influences on climate. If a variation of 1% in radiative input to the stratosphere has a significant effect, then an accretion producing $\delta L \approx 10^{-4} L_\odot$ in ultraviolet and X rays may have a large impact on terrestrial conditions, even though the change in the total heat balance is negligible. Thus, moderately dense interstellar clouds with $n(\text{H}_2) \approx 10^3 \text{ cm}^{-3}$ may have significant effects. The $\delta L \approx 10^{-2} L_\odot$ relationship discussed by McCrea³ would have a devastating effect on the Earth's upper atmosphere and perhaps cause ultraviolet fluxes hazardous to life¹⁸.

Dense interstellar clouds are well known because of the optical obstruction caused by dust grains about $0.2 \mu\text{m}$ in radius. Silicate and ice particles will survive solar radiation at distances from the Sun of a few $R_\odot$ and about 2 AU, respectively (see, for example, refs 18 and 19). Using 1% for the mass in grains per unit mass of H_2 , we estimate that once in a cloud the Earth will sweep up an average of about $6 \times 10^6 n(\text{H}_2) \text{ g yr}^{-1}$ of dust.

Our computations of the dynamics of infalling dust show that it will reach terminal velocity by the time it falls to about 90 km altitude, and that negligible ablation occurs. The residence time above the tropopause is at least 1 yr. This implies a dust loading of the terrestrial atmosphere of about 6×10^6 tonnes when the Solar System is in a cloud with $n(\text{H}_2) \approx 10^6 \text{ cm}^{-3}$.

This is about the level produced by volcanic activity, believed by some investigators²⁰ to produce perceptible climatic changes through alteration of atmospheric transparency and albedo. This dust may also provide nucleation sites and thereby influence precipitation. Both processes suggest that the accretion of interstellar dust by the Earth will lead to global cooling and glaciation. The frequency of passage through such dense clouds is probably very low, so that the significance of this effect is difficult to evaluate.

We estimate that during the lifetime of the Solar System the mass of dust grains (mostly silicon, magnesium, and oxygen) accreted by the Earth should have been about 10^{16} – 10^{18} g. Is there any evidence for their presence? These grains may contain freshly created, short-lived radioactive nuclei produced by supernova nucleosynthesis processes, and they may reveal themselves by exhibiting abundances appreciably greater than would be expected if the species had been decaying in isolation since the origin of the Solar System. Clayton²¹ has suggested that this may have already been observed in the detection of naturally occurring ^{244}Pu . Lindsay and Srnka²² have evidence for the accretion of interstellar dust by the Moon.

The processes proposed here have very complex implications for global weather patterns. We cannot at this time evaluate which, if any, will unquestionably affect the Earth's climate; however, astronomical influences upon climate seem to be far more complex and significant than considered previously. Begelman and Rees²³ have also considered some of the ideas discussed here.

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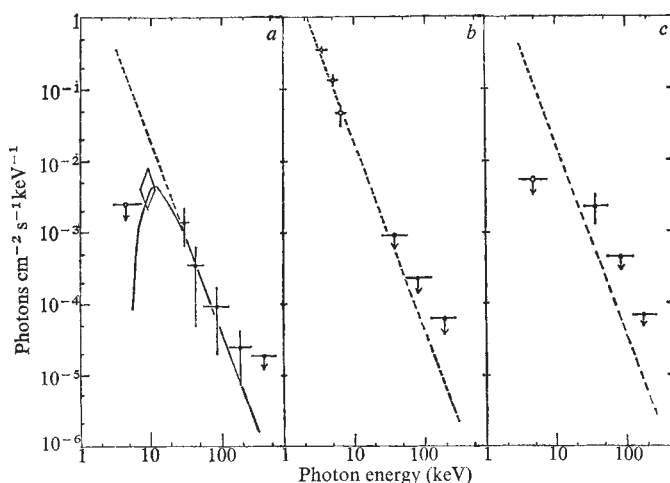


Fig. 1 Observed spectra of Cir X-1 before (a, February 10.5–14.5), during (b, February 16.5–19.5) and after (c, February 19.5–22.5) the outburst of 1976 February. $\circ$, RMC results¹; $\diamond$, OSO-7 quiet-state result²; $\bullet$, our ST results.

College hard X-ray scintillation telescope (ST) experiment (8° FWHM, 26 keV–1.2 MeV) completed a 4-d observation of this source and a strong signal was detected. For the following eight days, 1976 February 14–22, the source remained within the field of view, though at low sensitivity. Consequently only upper limits were obtained for the hard flux during the outburst and only a limited measure of the spectrum immediately afterwards. We have combined our results with the RMC results from reference 1 to obtain an overall view of the spectral behaviour of Cir X-1 during this outburst. We suggest that the large scale low energy X-ray outbursts of the 'super-variable' Cir X-1 may be interpreted as corresponding to the periodic removal of a dense screening gas to expose a relatively stable source with a power law spectrum.

For the period before the outburst the best fit to our data (see Fig. 1a) is found to be the power law

$$\frac{dN}{dE} = 14.6 E^{-2.7} \text{ photons cm}^{-2} \text{ s}^{-1} \text{ keV}^{-1}$$

Baity *et al.*² summarise OSO-7, balloon and rocket results at various times with the law

$$\frac{dN}{dE} = 4.8 E^{-2.6} \text{ photons cm}^{-2} \text{ s}^{-1} \text{ keV}^{-1}$$

although they have evidence for some spectral hardening as the overall intensity diminishes. Also this data shows intensity variations by a factor of >5 at energies below 10 keV. The power law given by Baity *et al.* also fits our data well. These authors provide a plot of the (7–11) keV flux at various times over the period 1971–1973 and note a relatively steady flux between outbursts of $(2\text{--}8) \times 10^{-3} \text{ photons cm}^{-2} \text{ s}^{-1} \text{ keV}^{-1}$. We therefore have included this data point in Fig. 1a.

During the outburst the soft X-ray flux increased by at least two orders of magnitude but there is no indication of any comparable increase in the flux of hard X-rays (Fig. 1b). Our experimental points for the period following the outburst, although less precise, indicate that the spectrum has not greatly changed (Fig. 1c). The ST results in Fig. 1a and the balloon data of Guo *et al.*³ from an earlier epoch do not fit a single temperature thermal spectrum. Wilson and Carpenter¹ suggest an absorption column density of $10^{22} \text{ atoms cm}^{-2}$ to explain their low energy data during the outburst. Margon *et al.*⁴ during a previous outburst find a sharp spectral cutoff, approximately consistent with this value.

Recently Kaluzienski *et al.*⁵ have reported that this source has a 16.5-d period with a phase consistent with the time of this

Hard X-ray observations of Circinus X-1 during an outburst

WHILE the Ariel V satellite was observing in the Circinus–Norma region an outburst was detected from Cir X-1 on 1976 February 15 by the rotation modulation collimator (RMC) experiment¹. One day before the outburst began the Imperial

outburst, though we note that an earlier event reported by Buff *et al.*⁶ does not fall on this period.

We interpret the totality of the data by assuming that there is an intrinsic power law source extending to $\lesssim 1$ keV and that varying amounts of cold gas change the cutoff from 2 keV to 12 keV thereby producing the outburst. A 12-keV cutoff requires a column density of 2.5×10^{24} atoms cm^{-2} of 'typical' interstellar matter and the resulting spectrum is shown as a solid line in Fig. 1a. One could imagine a compact object source immersed for most of the time in a dense, cold stellar wind or other 'cloud' of cold material which periodically thins to allow out the soft radiation. An eccentric orbit binary pair is an obvious model for the source.

Our results do not exclude the possibility that the absorbing cloud of gas could radiate by the blackbody mechanism with $kT \lesssim 7$ keV and the emerging spectrum at times of low intensity could be a superposition of this thermal radiation and intrinsic source spectra. The cold cloud is, however, more plausible because to prevent violation of the Eddington limit the hot cloud would have to be no larger than a neutron star. Cir X-1 may thus provide an extreme example of a source where large variations in the amount of surrounding material dramatically changes the spectral shape up to 10 keV or more.

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New H₂O maser near NGC7538

A NEW, strong, water vapour maser has appeared at the position of a very compact radio and infrared source near the H II region NGC7538. The radio source is NGC7538-B (infrared source IRS-1), the most intense of a group of three compact H II regions¹⁻⁴ at a distance of ~ 3 kpc from the Earth. The continuum source is optically thick below 20 GHz (ref. 5), and thus has one of the highest emission measures yet observed in a compact H II region. At 10 μm , it has one of the strongest silicate absorption features known⁶, and this source and the KL nebula in Orion are the only sources in the Galaxy which show formaldehyde in emission at 4.8 GHz (ref. 7). The area has been searched many times during the past 5 yr without the detection of this particular H₂O maser. Its sudden appearance may be related to the dynamics of the high-density shell surrounding the compact ionised region.

The observations were made on May 1 and 2, 1976, with the Effelsberg 100-m telescope, which at 22 GHz has a half-power beamwidth (HPBW) of $40'' \times 43''$ and, in the zenith, beam and aperture efficiencies of 35% and 15%, respectively, of the entire 100-m surface. The primary calibration source, NGC7027, had a zenith antenna temperature of 2.1 K and an assumed flux density of 5.9 Jy. The telescope pointing was determined relative to 3C84, 3C273, NRAO530 and 3C454.3, to an accuracy of $\pm 3''$. The mixer receiver had a system temperature of 1,000 K, single sideband, and was followed by a 384-channel autocorrelation spectrometer. With typical spectral resolution of 32 kHz (0.43 km s^{-1}) and integration time of 3 min, the root mean square (r.m.s.) noise was 0.6 K. The rest frequency of the H₂O transition was assumed to be 22.23507985 GHz, and the atmospheric extinction to be 15% in the zenith. All of the observations were made at night, in clear weather.

An area slightly larger than that shown in Fig. 1 was mapped with a grid of 36 points, separated by $20''$. Table 1 summarises the properties of the H₂O sources found in this map, and the inset in Fig. 1 shows their spectra. The southern H₂O source has been under observation since 1972 (refs 8-12), and it coincides with a 1,665-MHz OH maser¹³ for which no corresponding radio continuum or infrared source has yet been found. The newly discovered H₂O source coincides in position with the more northern OH source¹³⁻¹⁵ and with the very compact radio and infrared source. Although some previous workers using larger beamwidths than ours have listed the position of the northern OH source for their observations, it seems clear from the radial velocities that they were actually observing the southern H₂O maser. Two groups^{11,12} report limits of 3 Jy for any H₂O emission from the northern position. We conclude that the northern H₂O source has only recently appeared, or that it has reappeared after a long, quiet period.

The main component of this new H₂O source has a radial velocity of $-60.8 \pm 0.2 \text{ km s}^{-1}$ and a width to half-power of 0.6 km s^{-1} . Like many other H₂O sources, it has weaker components at other velocities. On our map, these components coincide in position to within $\pm 5''$ with the main feature at -60.8 km s^{-1} . Similarly, all of the velocity features in the spectrum of the southern H₂O source come from the same, southern position. Nowhere in the map could we find, to a limit of 6 Jy, the components at -112 km s^{-1} reported by Cato *et al.*¹¹.

The sudden appearance of the northern H₂O source may be related to other unusual properties of the compact H II region. A schematic model of the source (Fig. 2) is as follows.

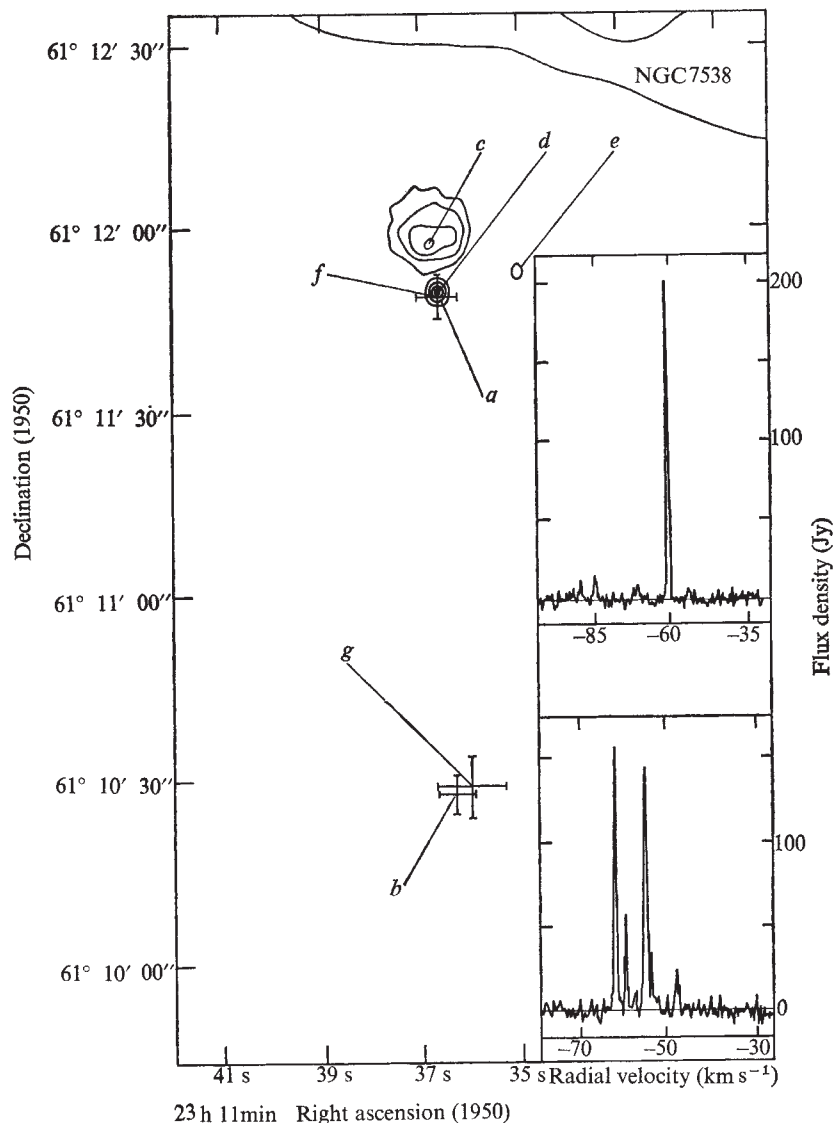
(1) The exciting star is probably of spectral type O6, since both the far infrared spectrum¹⁶ and the near infrared measurements⁶ correspond to a luminosity of $3 \times 10^5 L_{\odot}$. In contrast, the luminosity of the new H₂O maser is $< 10^{-4} L_{\odot}$.

(2) Surrounding the exciting star is a compact H II region with an angular size of $\sim 1''$. Its unusually high turnover frequency of 15-20 GHz (ref. 5) means that it is one of the most compact H II regions known, with an emission measure $> 10^8 \text{ cm}^{-6} \text{ pc}$, and an electron density $> 10^5 \text{ cm}^{-3}$. At 15 GHz (ref. 5), the H II region is 100 times weaker (0.5 Jy) than would be expected from its infrared luminosity. This may be evidence for a high density of dust within the H II region, which absorbs most of the ionising photons. An alternative explanation is that the exciting star has not yet reached the main sequence.

Table 1 Parameters of the H₂O masers near NGC7538

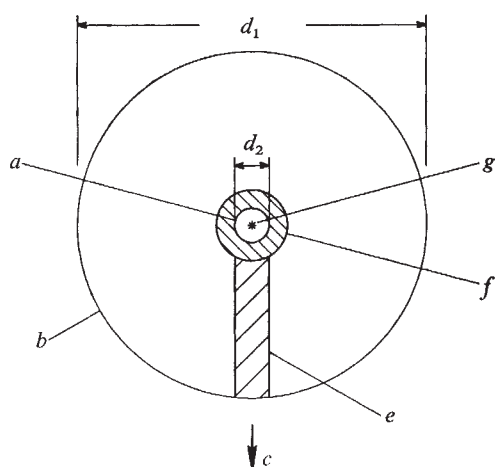
Source	Position (1950)	Radial velocity (line <i>a</i>) and line flux density (<i>b</i>)
New H ₂ O maser	23 h 11 min 36.6 ± 0.4 s 61° 11' 49" $\pm 3''$	<i>a</i> -89.4, -84.4, -70.7, -60.8 (± 0.2) <i>b</i> 13, 14, 10, 204 (± 4) (Jy)
Previously known H ₂ O maser	23 h 11 min 36.4 ± 0.4 s 61° 10' 28" $\pm 3''$	<i>a</i> -61.3, -58.6, -56.1, -54.1, -53.1, -46.6 (± 0.2) <i>b</i> 133, 48, 12, 124, 41, 24 (± 4) (Jy)

Fig. 1 Location of H_2O and OH masers and continuum sources in the vicinity of NGC7538: *a*, New H_2O source; *b*, previously known H_2O source; *c*, infrared source (IRS 2); *d*, IRS 1; *e*, IRS 3; *f*, OH 1665/1720 MHz; *g*, OH 1665 MHz. The positions and error bars for the northern H_2O and OH sources are the same. Radio contours are taken from Martin¹. Inset: Spectra of the northern (upper profile) and southern (lower profile) H_2O masers, obtained with a resolution of 0.43 km s^{-1} .



(3) Around the ionised region is a dense shell of dust and neutral gas of angular size $< 10''$. The visual extinction through this shell is mag 90, and the silicate absorption at $10 \mu\text{m}$ is

Fig. 2 Schematic model of the radio source NGC7538-B (= infrared source IRS 1). *a*, Compact H II region, $n_e > 10^5 \text{ cm}^{-3}$; *b*, shell of dust and neutral gas, $n_{\text{H}_2} > 10^5 \text{ cm}^{-3}$; *c*, $\sim 3 \text{ kpc}$ to Earth; d_1 , $\theta < 10''$, diameter $< 0.1 \text{ pc}$; d_2 , $\theta = 1''$, diameter $= 10^{-2} \text{ pc}$; *e*, zone of silicate absorption at $10 \mu\text{m}$ (visual extinction mag 90); *f*, transition zone between ionisation and shock fronts (location of H_2O masers); *g*, O6 star, $L = 3 \times 10^5 L_\odot$.



one of the strongest known⁶. From the silicate absorption, Willner⁶ has derived a hydrogen column density of $1.3 \times 10^{23} \text{ cm}^{-2}$, which with his size limit of $10''$ for the dust shell, implies a number density $n_{\text{H}_2} > 2 \times 10^5 \text{ cm}^{-3}$. This density is high enough to destroy the anomalous refrigeration of formaldehyde, and to explain why H_2CO is seen in emission from this source at 4.8 GHz (ref. 7). The agreement of density estimates from the silicate absorption, the H_2CO emission, and the radio continuum emission indicates that the compact H II region is ionisation bounded.

According to current ideas^{17,18} the ionisation front of the compact H II region is moving into the neutral material at a velocity of $\sim 10 \text{ km s}^{-1}$. The radius of the ionisation front is now $5 \times 10^{-3} \text{ pc}$, and the age of the source is $\leq 10^3 \text{ yr}$. Ahead of the ionisation front, by perhaps $1''$ on the sky, is a shock front, moving through the surrounding, dense, neutral matter. The H_2O maser features may arise in the heated region which has a kinetic temperature of 500–1,000 K between the ionisation front and the shock front. The velocity range of 30 km s^{-1} observed in the new H_2O source may correspond roughly to the difference between the radial velocities of the shock front and the exciting star. The H_2CO emission at 4.8 GHz and the OH masers at 1,665 and 1,720 MHz would come from somewhat cooler regions in the dense, neutral shell.

A fair amount of radio continuum, radio line and infrared data are now available for this source. Its geometry seems to be relatively simple, with a single exciting star surrounded by a compact H II region and a dense shell of dust. The object may

therefore be a useful prototype for the study of newly formed O stars and H₂O masers in general.

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The identity of lunar hydrolysable carbon

LUNAR soils treated with deuterated acid release a variety of deuterocarbon gases including CD₄, C₂D₆, C₂D₄, C₂D₂ and C₃ species¹⁻³. We now suggest that the principal hydrolysable carbon phase in lunar samples is carbon in solid solution in body-centred cubic iron (α -Fe). The yield of deuterocarbons (excluding C₃D₂) from a lunar soil is probably a quantitative estimate of this carbon.

The iron-carbon-oxygen system provides a useful basis for the evaluation of the iron phases in lunar samples although this does not mean that iron in lunar soil is necessarily formed by equilibrium gas phase reduction. We have examined synthetic alloys of carbon in α -Fe, together with some samples of cementite (Fe₃C), prepared by reduction of pure Fe₂O₃ in mixtures of CO and CO₂ at selected temperatures and 1 atm total pressure. The gases released by treatment with 38% DCl in D₂O were analysed by gas chromatography¹. The conditions of sample preparation, together with X-ray diffraction (XRD) data and the yields of deuterocarbons, are listed in Table 1.

The yields of deuterocarbons were low from samples which have been shown by XRD to contain cementite (A3, A5, 8224) and also from sample A1 which, according to the phase diagram, should contain this material. The majority of samples, indicated by XRD to be α -Fe, release considerable quantities of deuterocarbons (up to 0.6% by weight is hydrolysable carbon) the largest amounts being evolved from specimens prepared in conditions of high carbon solubility. An α -Fe (8225) prepared in non-carburising gases (H₂-CO₂ mixtures) had only a minimal amount of reactive carbon.

Our results were unexpected for two reasons: first, we obtained α -Fe samples in conditions which, according to the Fe-C-O phase diagram^{4,5}, should produce the γ phase; and second, the quantity of carbon present in the α -Fe is greater (up to 0.6 weight %) than the published solubility limit (0.02 weight %) of carbon in α -iron^{4,6}. Our starting materials were of much higher purity than those used to establish the phase diagram, and prepared samples were quenched⁷ after synthesis, which may allow solubility limits to be exceeded.

From the results, we conclude that iron carbides are unlikely to be the hydrolysable carbon phase in lunar soils. Carbon in solid solution in iron, probably associated with α -Fe, is a more likely candidate. All deuterocarbon gases liberated from lunar soils, with the exception of C₂D₂, can be accounted for by carbon in solid solution in iron phases. None of the synthetic samples released C₂D₂. In the investigation of lunar soils this species presumably results from hydrolysis of groups I and II metal acetylides which are the only carbides shown to release acetylene^{8,9}. Since the suggestion³ that the hydrolysable carbon phase in lunar soil might be the meteoritic carbide, cohenite (FeNi)₃C, the iron-carbon phase which is the precursor of the deuterocarbons has been referred to as carbide, "carbide", carbide-like or material reacting as carbide. On the basis of the present results, the use of the term carbide should be discontinued, as iron carbide seems to be essentially inert to acid attack¹⁰. The only fragments of true carbide to be identified in lunar fines are those recognised by microscopy¹¹. As only 1-2% of the carbon in samples of meteoritic cohenite could be converted to deuterocarbons by DCl, it has been suggested that almost all the total carbon present in lunar soils might be associated with iron¹². If the deuterocarbons released from lunar soil are considered as a quantitative yield of C in α -Fe, then only 15-25% of the total carbon is connected with free metal. The bulk of lunar carbon is presumably implanted solar wind atoms which exist in the silicate matrix of soil grains in an as yet unidentified chemical state.

There is considerable evidence that finely divided sub-micron-sized iron, which is ubiquitous in lunar soils^{13,14} is the α phase. Opaque inclusions ~ 150 Å in diameter in glassy agglutinates, give a diffraction pattern consistent with α -Fe (ref. 15). Ferromagnetic resonance data¹⁶ imply that α -Fe has a low nickel content. The Curie points determined for iron in lunar soils^{17,18} (~ 770 °C) are greater than those for γ -iron and cohenite, which are less than 600 °C and 215 °C respectively¹⁹. The ratios of C₁ to C₂ deuterocarbons obtained by the Berkeley group^{8,20} from lunar soils are similar to the values listed for C in α -Fe in Table 1.

Using data for carbon as CD₄ from 15 lunar soils¹ and values obtained by ferromagnetic resonance spectroscopy^{13,21} for the abundance of finely divided metal in aliquots of the same samples, the concentration of carbon in the α -iron in lunar material can be calculated as 0.27-0.77% by weight. The calculated values are in excess of the solid solubility limits of C in α -Fe but are similar to the values obtained for our synthetic α -Fe samples.

The processes of formation of lunar metal and/or the incorporation of carbon evidently allow the normally accepted solid solubility limits to be exceeded. Perhaps iron grains ~ 150 Å in diameter contain insufficient crystal boundaries and defects to promote the crystallisation of carbide. As already stated, it is unlikely that iron in lunar soils was formed in equilibrium conditions. We have tried to reduce silicate glasses of lunar composition in conditions similar to those used for the preparation of α -Fe samples. In all cases, the amounts of CD₄ released from reduced glasses were very small: < 0.05% of the glass had been reduced to Fe metal, even though reaction times of up to 24 h were used. The presence of silicate is known to require more strongly reducing conditions for iron formation²². The subsolidus reduction process suggested¹⁷ as the origin of iron in lunar soil may, in fact, be appropriate only to major impacts which produced highly metamorphosed breccias containing coarse grained iron and very little hydrolysable carbon²³.

Of the mechanisms which have been suggested for the formation of metallic iron in the lunar regolith, only preferential sputtering by the solar wind has been shown to be both theoretically feasible²⁴ and practically possible²⁵.

Table 1 Deuterocarbons released by DCl dissolution of synthetic iron-carbon phases

Sample	Temp. (°C)	Preparation CO in reducing gas* (%)	Deutero-carbon yield $\mu\text{g C per g}$				CD ₄ /C ₂ D ₄ + C ₂ D ₆	XRD
			CD ₄	C ₂ D ₄	C ₂ D ₆	Total		
A1	700	80	346	NM	NM	346	—	No peaks
A3	800	90	ND	ND	ND	ND	—	Fe ₃ C
A4	900	90	4,891	493	1,071	6,455	3.13	α
A5	900	96	1,088	NM	NM	1,088	—	$\alpha + \text{Fe}_3\text{C}$
8222†	800	79	2,488	527	1,230	4,245	1.42	α
8224	800	100	ND	ND	ND	ND	—	Fe ₃ C
8225	800	0§	49	ND	6	55	8.36	α
8226	800	86	1,433	285	729	2,447	1.41	α
8227	800	84	3,976	149	1,002	5,127	3.45	α
8228	900	86	313	41	164	528	1.53	α
8229	900	90	1,621	733	984	3,338	0.94	α
8231	930	90	1,196	463	722	2,381	1.01	α
8232	900	80	908	186	437	1,531	1.46	α
8233	800	72	947	149	310	1,406	2.06	α
8234	700	70	2,327	530	1,368	4,225	1.23	α
8235	900	73	113	152	53	318	0.55	α

* Balance due to CO₂, total pressure 1 atm.

† Average of four analyses.

§ Non-carburising reactions conditions 1:1 mixture of H₂ and CO₂.

NM, Not measured; ND, not detected.

Carbon could form solid solutions in surface layers of α -iron produced by sputtering in one or more of the following ways: (1) direct implantation of solar wind carbon into metal—this process is capable of producing solid solutions of carbon in α -iron in excess of solubility limits²⁶; (2) under the influence of sputtering, the particle surface will be eroded and iron at the surface will gradually reach carbon previously implanted by the solar wind²⁷; (3) thermal events associated with agglutination could induce migration of solar wind carbon from implantation sites to the surface iron²⁷, and micrometeorite impacts may release carbon species which carburise metal on the surfaces of adjacent grains¹⁰. All of these suggested processes for carbon could be applicable to the accumulation of chemically bound nitrogen in lunar samples²⁸. The increased solubility of nitrogen in iron⁹ would account for the greater retentivity of nitrogen^{28,29}.

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Implications of metal dispersion from submarine hydrothermal systems for mineral exploration on mid-ocean ridges and in island arcs

METAL enrichments in sediments on open ocean mid-ocean ridge segments and in island arcs have been known for some time^{1–4}, but, to date, no deposits have been found which are rich in copper, zinc and other heavy metal sulphides to a degree comparable to those in the Red Sea. The fact that concentrated brines were thought to have played an essential role in the formation of the Red Sea metalliferous sediments may have inhibited the search for similar deposits elsewhere, because brines of the Red Sea type are unlikely to occur in the open ocean. The discovery at Matupi Harbour, New Britain, however, that high salinity is not a prerequisite for metal enrichment in hydrothermal solutions⁵, indicates that any area of submarine volcanic activity has a potential for high grade metalliferous sediment formation given suitable depositional conditions. In this report the dispersion of metals from submarine hydrothermal sources

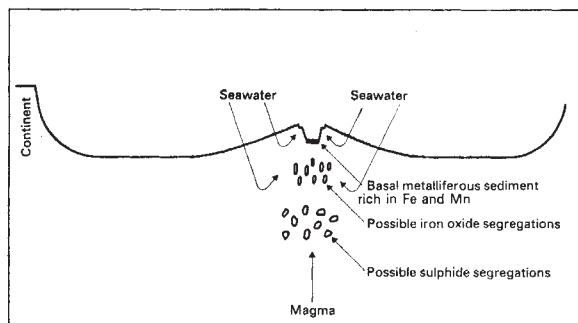


Fig. 1 Hypothetical vertical sequence of precipitates on mixing of ascending metal-bearing hydrothermal solution with seawater beneath mid-ocean ridge crests in the open ocean. This sequence occurs horizontally on the sea floor around hydrothermal centres in restricted environments like the Atlantis II Deep in the Red Sea and in shallow water volcanic areas, indicating that the nature of the environment of deposition controls the initiation of the depositional process.

is discussed, and its usefulness in developing guidelines for undersea mineral exploration in volcanic areas is considered.

Metalliferous sediments on mid-ocean ridges and in island arcs are thought to form largely as a result of hydrothermal leaching of the oceanic crust by circulating seawater, which in turn becomes both acid and reduced^{6,7}. Precipitation of the metals so derived gives rise to deposits which are characteristically rich in iron oxides and sometimes in a variety of other metals such as manganese, copper, zinc and lead; and occasionally, as in the Red Sea, include metal sulphides of localised distribution and very variable composition. It is the latter which are of potential economic value.

Evidence that mid-ocean ridge sediments vary in composition over short distances in the open ocean, and thus may locally be of high grade has been obtained on the Mid-Atlantic Ridge at 45°N (ref. 8). The chemical variations, particularly in the relative dispersion of iron and manganese, were thought to be related partly to the proximity of the samples analysed to submarine hydrothermal sources of the metals and partly to their selective precipitation on mixing with seawater. Unfortunately, the implications of these variations, in terms of geochemical exploration, for metalliferous sediments could not be evaluated because the location of the presumed hydrothermal outlets on the sea floor were not known. More recently, the selective dispersion and precipitation of iron and manganese from a known hydrothermal source has been noted in the FAMOUS area on the Mid-Atlantic Ridge (X. Le Pichon, personal communication), and hydrothermal manganese deposits have been found on the Mid-Atlantic Ridge at 26°N (ref. 9). Nevertheless, there has been no systematic study published to date of metal dispersion from a submarine hydrothermal source in the deep ocean. Fortunately, however, the likely nature of deep-sea hydrothermal metal dispersion can be deduced from studies of submarine hydrothermal systems in the Red Sea and off Santorini. On this basis a model has been constructed which attempts both to predict the most favourable environments for high grade metalliferous sediment formation on the ocean floor and to aid in the location of the sediments.

Metalliferous sediments of hydrothermal origin have been studied in both the Red Sea, and off the volcano of Santorini in the Cyclades Volcanic Arc in the eastern Mediterranean Sea. In each case there is a dispersion halo of metals around the hydrothermal source, showing, among other things, the selective dispersion of manganese from iron^{10,11} (Fig. 1). A similar sequence has been observed around hydrothermal springs debouching into Matupi Harbour, New Britain¹². These variations probably reflect the well known separation of manganese from iron with changing pH and Eh (ref. 13). Poorly oxidised or reduced

acid hydrothermal solutions precipitate the metals selectively on mixing with more alkaline oxidising seawater. Metals other than manganese and iron also exhibit a fractional precipitation in submarine hydrothermal systems. In the Atlantis II Deep of the Red Sea, for example, the first formed precipitates on discharge of the hydrothermal solutions are heavy metal sulphides which occupy a restricted area of the Deep adjacent to the hydrothermal vents¹⁴. Similarly, sulphides occur underneath the sediments rich in iron oxides near the hydrothermal vents in the caldera of Santorini¹⁵, and immediately adjacent to fumaroles off the Island of Vulcano in the Eolian Archipelago, Western Mediterranean¹⁶. It seems, therefore, that a model for the complete sequence of precipitates on mixing of hydrothermal solutions with seawater would include the fractional dispersion and precipitation of metal sulphides, followed by iron silicates and/or iron oxides, and finally manganese oxides.

This complete sequence of precipitates has not been reported to date on mid-ocean ridges in the open ocean. The iron- and manganese-rich metalliferous sediments characteristic of such ridges, however, are compositionally similar to the widely dispersed iron and manganese oxides around the Atlantis II Deep where the complete sequence is observed. This suggests that the former may be the equivalent of the sediment in the outer zone of the Atlantis II dispersion halo. If this is so, the sulphide deposits which the model predicts should be present in association with the iron and manganese oxides may occur either in restricted locations near to the hydrothermal vents or within the oceanic crust below the oxide deposits. The general physicochemical nature of seawater over mid-ocean ridge crests in the open ocean would favour the latter alternative in most cases. Oxidising seawater can be expected to penetrate fractures and fissures in the upper part of the oceanic crust leading to mixing with ascending hydrothermal solutions and the precipitation of metal sulphides before the solutions reach the surface (Fig. 1). Disseminated sulphides within rocks of the oceanic crust have been reported by several authors¹⁷⁻¹⁹. Special conditions leading to a reducing environment at elevated temperature, such as occur in the Atlantis II Deep of the Red Sea, would be required to delay sulphide precipitation until the hydrothermal solutions debouch on to the sea floor.

If sulphides are largely precipitated from submarine hydrothermal solutions within the oceanic crust, where else on the sea floor other than in the Red Sea and in small restricted embayments around volcanic islands might they be expected to form? One obvious locale is within the deep fracture zones which cut mid-ocean ridges in all the oceans and which often expose the oceanic crust to considerable depths. Deep hydrothermal circulation under these fracture zones coupled with the possibility of locally reducing conditions in narrow deep basins, could lead to the precipitation of heavy metal sulphides. Bonatti *et al.*²⁰ have explained high heat flow in some fracture zones on the basis of subsurface hydrothermal circulation, and have reported the occurrence of pyrite concretions of supposed hydrothermal origin in the Romanche Fracture Zone, Equatorial Atlantic. In addition, Scott *et al.*²¹ have reported the occurrence of manganese deposits of probable hydrothermal origin in the Atlantis II Fracture Zone in the North Atlantic. An additional likely locale for heavy metal sulphide precipitation is in the basins associated with island arcs. These can be both volcanically active²² and sufficiently topographically diverse to possibly lead to a locally restricted circulation and reducing conditions favourable for sulphide deposition. Ancient marginal seas associated with island arcs are thought to have been the environment of formation of some massive sulphide deposits now exposed on land²³. Iron- and manganese-rich sediments similar to those on mid-ocean ridges have been found in association with the island arc system in the

south-western Pacific Ocean²⁴, and submarine sulphide deposits may also be present.

It is evident from the above that mid-ocean ridge fracture zones and island arcs warrant more attention in the search for ocean floor metalliferous sediments. The dispersion model discussed here may additionally be of use in locating such deposits. The dispersion halo of manganese around the Atlantis II Deep deposit is up to 20 km in diameter even though the sulphide deposit itself is much smaller²⁵. Thus in designing a grid sampling program to locate heavy metal sulphides on the ocean floor, one could look initially for manganese dispersion haloes on a wide-spaced sampling pattern, and, if located, design a smaller grid to search the vicinity for heavy metal sulphides. Tests of the model as an exploration tool are currently being conducted around the TAG hydrothermal field on the Mid-Atlantic Ridge at 26°N, and in a fracture zone transecting the East Pacific Rise at 9°S.

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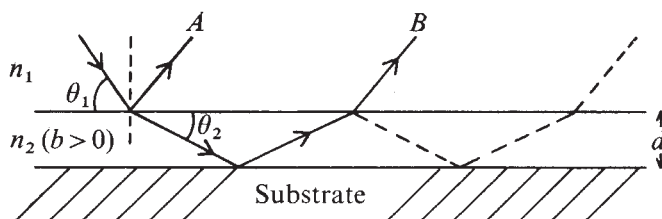


Fig. 1 Reflection of neutrons from a plane parallel plate. n_1 and n_2 are the refractive indices in air and in the thin film respectively. (Note that for neutrons $\theta_2 < \theta_1$, in contrast to the case for light.) Angles are greatly exaggerated for clarity.

Since b may be positive or negative, n_2 may be larger or smaller than 1. From classical optics¹, the path difference for beams A and B can be shown to be

$$\Delta = 2d(\theta_1^2 - \theta_c^2)^{1/2} \quad (3)$$

As there is no phase change for the reflection of neutrons, the condition for constructive interference is given by

$$\Delta = m\lambda \quad (\text{where } m = 0, 1, 2, \dots) \quad (4)$$

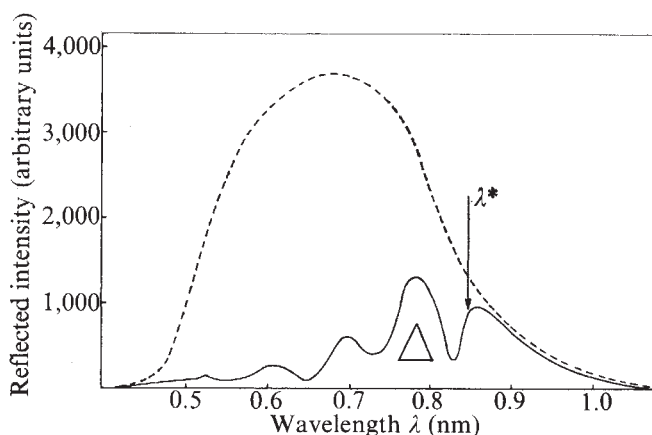
For positive values of b , $m = 0$ corresponds to the limit for total reflection, and interference fringes will only be observed when $\theta_1 > \theta_c$; for negative values of b , interference is possible at all glancing angles. From equations (3) and (4) we see that at a given angle of reflection θ_1 , intensity maxima will occur when

$$\lambda = \frac{2d}{m}(\theta_1^2 - \theta_c^2)^{1/2} \quad (5)$$

This analysis determines only the relation between θ_1 and λ at the intensity maxima, and is unaffected by multiple reflection effects.

Interference fringes were observed by reflecting a narrow (1-mm wide), well collimated (divergence = 1.5×10^{-4} rad) neutron beam from thin metallic films evaporated on to plane glass substrates. The film materials used (Be and a magnetised $\text{Co}_{0.5}\text{Fe}_{0.5}$ alloy) have critical glancing angles greater than that of glass, thus ensuring that substrate reflections do not contribute to the interference peaks. At angles of incidence $\theta_1 > \theta_c$ interference fringes will be observed at wavelengths which satisfy equation (5). For the magnetised material

Fig. 2 Interference fringes measured for neutron reflection from a magnetised $\text{Co}_{0.5}\text{Fe}_{0.5}$ thin film. The wavelength spectrum of the incident beam is shown dashed (not to scale) for comparison. λ^* is the critical wavelength (equation (1)) above which all neutrons are totally reflected at this incident angle ($\theta_1 = 1.5 \times 10^{-2}$ rad). Δ represents the wavelength resolution.



Observation of the interference of neutrons reflected from thin films

DURING measurements of the reflection of neutrons from metallic thin films, we have observed an optical interference phenomenon analogous to the interference of light reflected from plane parallel plates; the experimental arrangement is the neutron analogue of the Lummer-Gehrcke interferometer¹.

A simple application of Snell's Law² predicts the essential features of the effect, which is illustrated in Fig. 1. We recall that neutrons of wavelength λ incident at glancing angles θ_1 , on a material characterised by a mean coherent scattering length b and with a density of scattering centres N , undergo total reflection when $\theta_1 < \theta_c$ where the critical glancing angle θ_c is given by³

$$\theta_c = \lambda (Nb/\pi)^{1/2} \quad (1)$$

The refractive index n_2 of the film material may be written as

$$n_2 = 1 - \theta_c^2/2 \quad (2)$$

b (equation (1)) is replaced by $(b+p)$ and $(b-p)$ for the two neutron spin states in the incident beam, where p is a magnetic scattering length, and where $|b| \simeq |p|$ it is only for the (+) neutron spin state that n_2 differs significantly from unity. This situation exists for the magnetised alloy we used, therefore the neutrons detected in the interference peaks for this thin film are also spin polarised.

The experiment consisted of reflecting a polychromatic incident beam (mean wavelength $\lambda_0 = 0.7$ nm) at a fixed angle θ_1 , and analysing the wavelength spectrum of the reflected beam by the neutron time of flight method. The measurements were carried out on the IN11 instrument at the High Flux Reactor, Institut Laue-Langevin, Grenoble; the angular resolution $\delta\theta/\theta_1$ was 1% and the wavelength resolution $\delta\lambda/\lambda_0$ was 5%.

Figure 2 shows the time of flight data observed for a magnetised $\text{Co}_{0.5}\text{Fe}_{0.5}$ film of thickness 100 ± 15 nm (as measured by a quartz crystal thickness monitor) at $\theta_1 = 1.5 \times 10^{-2}$ rad. Fitting equation (5) to the measured fringe positions gave a 'best-fit' thickness $d = 88 \pm 5$ nm, in agreement with the thickness measured during the evaporation of the film.

The wavelength spectrum of the incident beam (see Fig. 2) limited our observation to four orders of interference. For the beam divergence used, and assuming that the film thickness is completely uniform, the Heisenberg Uncertainty Principle requires the neutron coherence length in this example to be at least 70 nm; it should therefore be possible to observe interference fringes to much higher orders than are reported here.

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Optical methods to select diamonds of very high lattice perfection

WE describe here recent developments which permit the selection of diamonds very free from strain by purely optical inspection. Figure 1a shows the edge of a diamond slab which chipped while being polished, and Fig. 1b the same slab viewed between crossed polars¹. The birefringent pattern indicates a region of strain centred near the crack, and a dark area where the chip has come away relieving the strain. This type of strain birefringence is, of course, well known, and by carefully selecting diamonds on the basis of their birefringence it is possible to obtain stones for tools with a superior resistance to chipping. For example, the six similar tools in a wear experiment described by Casey and Wilks² were chosen in this way, and wore steadily without any chipping. Bell, Wilks and Wilks³ have described how the resistance of a diamond to cracking and chipping may be estimated by measuring the load on a small diamond sphere required to produce a ring crack on a flat diamond surface. In these experiments, measurements on a diamond slab chosen to be free of birefringence gave consistent values of the fracture load which were about twice as high as those on another slab which showed marked birefringence.

Minimal birefringence is an obvious requirement for strain free stones, but a more critical selection can be made

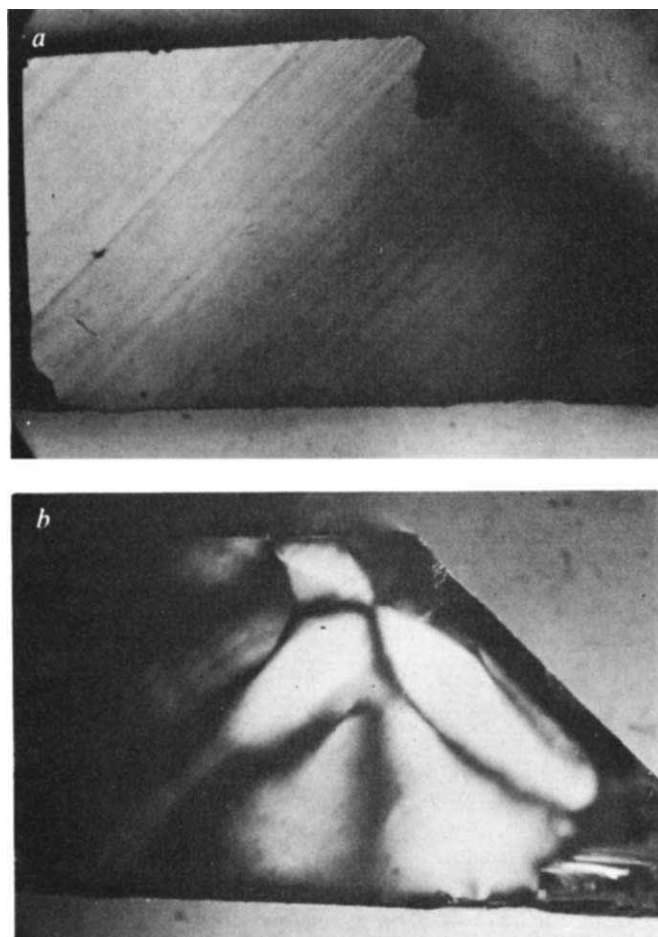


Fig. 1 The polished face of a slab of diamond viewed *a* in incident light, *b* between crossed polars ($\times 36$).

by inspecting the natural octahedron surfaces of a diamond using phase contrast or Nomarski interference techniques. This method had its genesis in an observation⁴ of a good quality gem octahedron which exhibited a small group of very shallow pyramidal depressions, or trigons, situated somewhat away from the centre of each face. The fact that the three lines joining the centres of opposite groups of trigons met approximately in the same internal region of the diamond, suggested the presence of an inclusion, from which dislocations spread out to the surface, where they gave rise to the trigon etch features. This interpretation was subsequently confirmed by Frank and Lang⁵ using X-ray topography. The presence of trigons on a natural face is thus a further indication of internal strain, and subsequent experiments by Wilks and Wilks⁶ showed a marked correlation between the density of trigons and the degree of birefringence. A third indication of lattice imperfection is that carefully polished cube faces of diamond containing impurities deposited on octahedral planes during growth often exhibit rectangular markings or traces where these planes of impurities intersect the surface⁷. In fact, we have never observed any such traces on diamonds selected to have minimum birefringence and freedom from trigons⁸.

Perhaps the most refined selection of strain free diamonds yet made is our recent selection of diamonds for targets for the production of polarised photons in the 5-GeV electron synchrotron NINA of the SRC Daresbury Laboratory, as described by Jackson⁸. To produce polarised photons, the diamond must be oriented so that the synchrotron beam makes an angle of $\sim 2 \times 10^{-3}$ rad with a set of lattice planes in the diamond, with an accuracy of better than 10^{-4} rad. It follows that any local misorientations of the

diamond lattice must be small compared with 2×10^{-3} rad, but natural diamonds frequently show lattice curvature of this order. The first stone selected by our optical inspection had a curvature as indicated by an X-ray rocking curve analysis of $< 5 \times 10^{-5}$ rad, and was the first diamond to give a satisfactory beam of polarised photons at Daresbury⁸. Subsequently two more diamonds selected in the same way have met the same stringent requirement of producing a satisfactory beam of photons. Of course, to obtain such a degree of perfection it is necessary to use high quality optics and to make the selection from a large sample of diamonds.

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Ordered states in systems of macroscopic particles

ONE of the most striking observations of systems consisting of macroscopic particles ($\sim 1 \mu\text{m}$ in diameter) dispersed in a uniform background, is that in certain circumstances the particles can form themselves into an ordered or solid-like array. The importance and significance of these structures to a wide range of materials, including virus suspensions, soils, lacquers and latices, was recently discussed quite extensively by Efromov¹. In recent years this has also been the subject of considerable experimentation on well characterised polystyrene latices²⁻⁴. We have developed a theoretical model for a stable colloidal dispersion⁵ in which this disorder-order transition in a system of macroscopic particles is treated as the analogue of the fluid-solid transition in molecular materials. This theoretical approach shows that this ordering is a consequence of the repulsive forces between the particles. We show the more detailed features of the structure of the disordered and ordered phases of systems of colloidal or macroscopic particles.

Briefly, the model replaces an infinite system of interacting particles by a small (32 or 108 particles) periodic system. The interaction⁶, consisting of a combination of van der Waals' attraction and double layer repulsion, is assumed to operate between all possible pairs of particles in the system. The properties of the system, such as excess pressures, internal energies and radial distribution functions, are then obtained by Monte Carlo averaging over the likely configurations of the particles. The results obtained are very similar to the measured properties of aqueous polystyrene colloids^{7,8}; thus the model is at least qualitatively correct.

One significant advantage of the Monte Carlo model, over perhaps more expedient but less exact approaches⁷, is that it enables one to obtain much finer details of the structure of a system than can be obtained experimentally. For example, one cannot only calculate the experimentally accessible radial distribution function, but also the higher order distribution functions^{8,9} and, even more interestingly, typical configurations can be displayed with the Monte Carlo model¹⁰.

We have chosen a colloidal system consisting of spherical particles of diameter 595 nm with a surface potential of

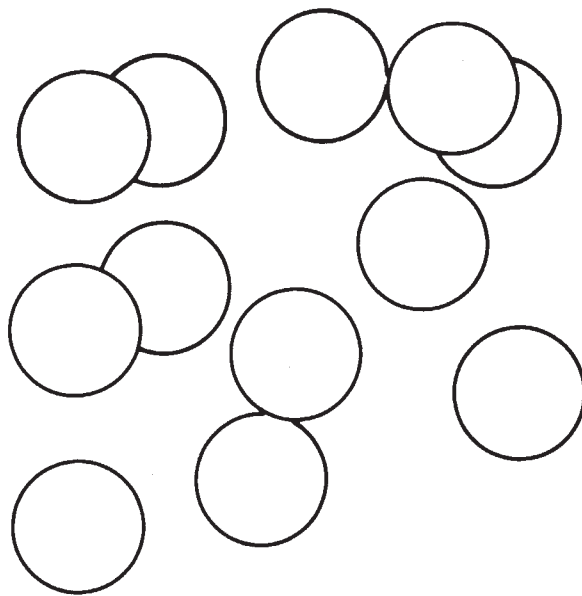
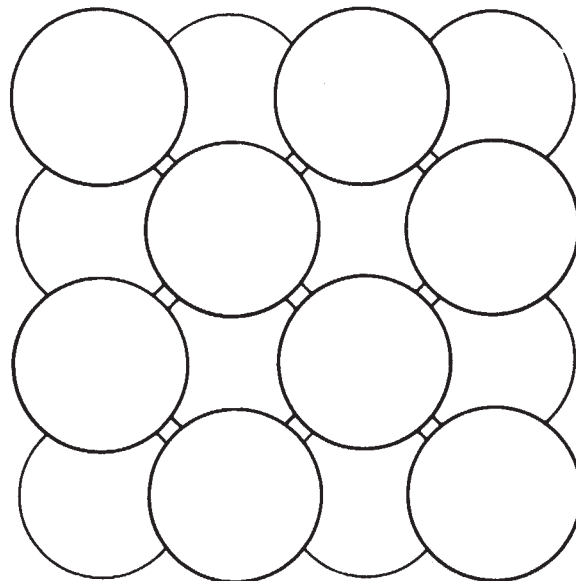


Fig. 1 Two-dimensional projection of a typical Monte Carlo (MC) configuration for a volume fraction $\phi = 25\%$ and concentration of monovalent 1:1 background electrolyte $n = 0.5 \text{ mol m}^{-3}$.

60 mV, dispersed in an aqueous electrolyte (relative permittivity of 80) at room temperature⁵. The results of the Monte Carlo model at electrolyte concentrations of 0.5 mol m^{-3} and 0.05 mol m^{-3} will be discussed.

For the higher electrolyte concentration the particles form an ordered array (face-centred cubic (fcc)) for percentage volume fractions, ϕ , in excess of 43%. This may be seen from the radial distribution functions, $g(r)$, calculated for the respective volume fractions at this electrolyte concentration⁵. To demonstrate what this gross structure indicator, $g(r)$, is showing, we display typical particle configurations for $\phi = 25\%$ and $\phi = 50\%$ in Figs 1 and 2, respectively. (In these diagrams we have drawn only those particles whose centres lie in a slice of thickness approximately equal to one particle diameter.) As may be seen, the system of particles at the higher volume fraction is indeed ordered and nearly conforms to a perfect fcc structure

Fig. 2 Two-dimensional projection of a typical MC configuration for $\phi = 50\%$ and $n = 0.5 \text{ mol m}^{-3}$.



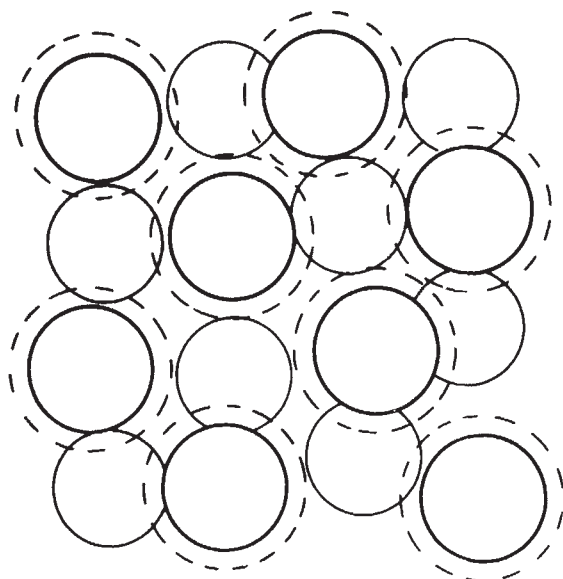


Fig. 3 Two-dimensional projection of a typical MC configuration for $\phi = 25\%$ and $n = 0.05 \text{ mol m}^{-3}$. The dashed circles represent the effective particles.

with a particle surface to surface separation as one would expect in a microscopic crystal. On the other hand, no such ordering is evident for the system of particles at the lower volume fraction.

At the lower electrolyte concentration (0.05 mol m^{-3}) the particles change to an ordered state at $\sim \phi = 25\%$. Figure 3 shows the distribution of particles for this case for $\phi = 25\%$. Again, an almost regular fcc arrangement is indicated but now with a particle surface to surface separation of $\sim 280 \text{ nm}$. As one lowers the background electrolyte concentration the particles order themselves at progressively lower volume fractions.

The reason for this behaviour is that the bare particle diameter is not a good indicator of the real size of the particle. Rather, the effective size of the particle is that of the particle plus the effective thickness of the attached double layer, the latter being a measure of the range of the repulsive potential acting between the particles. In terms of this effective diameter the properties of the system, including the disorder-order transition, can be very simply accounted for by means of a hard sphere model¹¹. Some simple criteria exist for calculating the effective hard sphere diameter and Fig. 3 shows the effective particles superposed on the real particles. The effective volume fraction of the system is $\sim 50\%$ with the mean surface to surface separation of the effective particles $\sim 25 \text{ nm}$ (see Fig. 2).

One final point to be made is that the exact lattice type observed for the ordered phase will depend strongly on the interparticle potential, particularly on its range.

In conclusion, it is hoped that this short article has demonstrated the power of machine methods to explore and display the structure of physical systems in general, and that it will be used more in the future to help visualise and understand the fascinating properties of colloidal dispersions, in particular.

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Polymer effects on microjet impact and cavitation erosion

THERE is a growing interest in using high molecular weight polymers as additives in various fluid-engineering systems. These activities are mainly stimulated by the anomalous drag reducing characteristics some high polymers exhibit in turbulent flows¹. Early experiments also showed that polymeric additives suppress flow-generated cavitations^{2,3}. An interesting question may therefore be raised as to the effects of high molecular weight polymers on cavitation erosion. We report here attempts made to examine such effects on static vibration-generated cavitation and flow cavitation.

It is generally accepted that in cavitation erosion the main damage results from the collapse of single cavitation bubbles near the solid surface. In such a collapse an inward moving microjet is always formed against the solid surface⁴ and the damaging effect of the microjet with velocity as high as 130 m s^{-1} impinging on the surface⁵ has been confirmed⁶. It was proposed earlier⁷ that polymer viscoelastic effect might introduce very high stresses by stretching the fluid, and consequently the formation of the microjet might be retarded. It was not, however, realised until recently that the development of such high stresses requires substantial flow time⁸. In the case of a microjet, although the jet velocity increases rapidly to apply a strong stretching to polymer molecules, the flow time involved is only $\sim 10^{-6} \text{ s}$, considerably shorter than polymer relaxation times ($\sim 10^{-3} \text{ s}$). Therefore one may have to expect that any polymer effect on microjet formation and thus cavitation erosion would be minimal.

Recently, Ashworth and Proctor⁹ reported the results of standard vibratory tests in solutions of a hydrolysed polyacrylamide, where the cavitation erosion rate is increased. They also showed that the viscous effect was not responsible for the increased damage because, in a water-glycerol mixture, the cavitation damage was actually decreased greatly. While no indication of the possible mechanism was given, the result of increased damage was considered to be analogous to that of enhanced jet-cutting in polymer solutions¹⁰. Some remarks will now be offered with regard to this proposed analogy.

First, the high speed liquid jets for jet-cutting operations have very high Reynolds numbers and are therefore turbulent. In these applications fluid momentum is converted into stagnation pressure to erode away the solid material. The effect of polymer in this case is mainly to introduce improved local stability to prevent small scale jet breakup. The result is that the turbulent jet possesses less violent appearance, higher velocity and longer throw¹¹. In the case of cavitation microjet, however, because of their extremely short lifetime and small physical size, instability-type disturbances would not have enough time to grow so the laminar flow prevails. The damage caused by the microjets is perhaps of an impact nature, characterised by the water-hammer pressure, estimated⁵ to be as high as 600 atm. This most impressive stress is sufficient to crack the solid to initiate subsequent material removal, and is in direct contrast to the gradual 'chew-away' actions in turbulent jet-cutting. This consideration is further shown by the liquid viscous effects. In the case of jet-cutting, Semerchan¹² demonstrated that a systematic increase in viscosity causes the jet forces to increase accordingly. But in the more viscous glycerol-water mixture cavitation erosion rate was lower as compared with the water

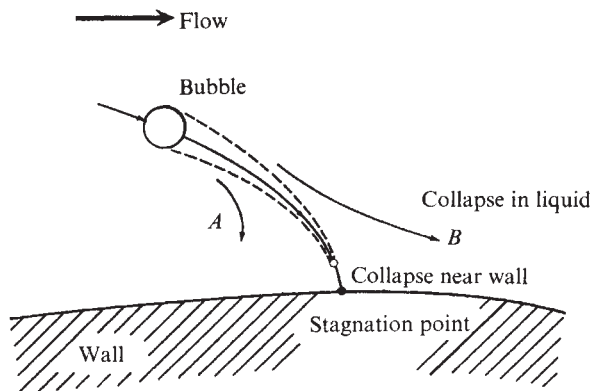


Fig. 1 Schematic representation of collapsing travelling bubbles approaching a stagnation point.

case⁹. This clearly suggested that the nature of the microjet is different from that of the turbulent jets for jet-cutting and the erosive activity of microjets is less intensive in a more viscous environment.

The explanation for the observed increase in cavitation erosion rate in polymer solution may still have its origin in polymer viscoelasticity. A deformational process in polymer solutions may be characterised by the relative scale between the duration of the deformation process and the polymer relaxation time. If the deformation time is sufficiently long, the polymer molecules adjust readily to the new deformed state. But if the duration of the deformation is much shorter than the polymer relaxation time, not enough time is allowed for the polymer molecules to adjust themselves and the material can only respond in a 'solid-like' elastic fashion. Since in the case of cavitation microjet the time scale is only $\sim 10^{-6}$ s and much shorter than polymer relaxation time ($\sim 10^{-3}$ s), it is expected that the polymer jet impinges on the wall with more solid-type impact than the water case. This additional impact stress may be briefly developed.

Consider a simple viscoelastic model for rapid deformation¹³ where the total stress τ is

$$\tau = -pI + GB^{(1)}\Delta t + 2K[B^{(1)}\Delta t]^2 + \frac{G}{2}B^{(2)}\Delta t^2 + \dots$$

where $B^{(n)}$ are deformation tensors defined as

$$B_{ij}^{(1)} = \frac{\partial v_i}{\partial x_j} + \frac{\partial v_j}{\partial x_i}$$

$$B_{ij}^{(n+1)} = \frac{DB_{ij}^{(n)}}{Dt} - B_{mj}^{(n)}\frac{\partial v_i}{\partial x_m} - B_{im}^{(n)}\frac{\partial v_j}{\partial x_m}$$

G and K are the material moduli, and Δt represents the very short duration of deformation. For an axial motion with velocities

$$v = (\gamma x_1, -\frac{\gamma}{2}x_2, -\frac{\gamma}{2}x_3)$$

it can be readily shown that

$$\tau_{11} = -p + 2G\gamma\Delta t + (8K - 2G)\gamma^2\Delta t^2$$

For extremely small Δt ($\sim 10^{-6}$ for the interest here) as compared with polymer relaxation time ($\sim 10^{-3}$), we may approximate

$$\tau_{11} = -p + G\gamma\Delta t$$

A modified Bernoulli equation may be written as

$$\frac{1}{2}\rho U^2 - \tau_{11} = \frac{1}{2}\rho U^2 + p - 2G\gamma\Delta t = p_\infty$$

where p_∞ is the pressure in a quiescent reference state of uniformity. Compared with the Newtonian case

$$\frac{1}{2}\rho U^2 + p_N = p_\infty$$

one observes that the difference represents an additional impact stress (or pressure)

$$\Delta p = p - p_N = 2G\gamma\Delta t$$

This term, which may be responsible for the increased cavitation erosion effect in polymer solution, is proportional to the elasticity of the liquid represented by an elastic modulus G , which normally scales with polymer concentration c and molecular weight like $c[\eta]$ if $[\eta]$ is the polymer intrinsic viscosity. This is in agreement with the observed result that the cavitation erosion rate is slightly higher in 1,000 p.p.m. solution than in 100 p.p.m. solution⁹.

Finally, while the results of Ashworth and Procter were obtained in a static vibratory-test system, it is noted that the cavitation fields occurred in most actual engineering applications are involved in a flow system. It is known⁶ that only one out of 10^4 – 10^{10} cavitation bubbles that are swept into a stagnant flow region seems to collapse adjacent to a solid surface and actually produces a damage pit (Fig. 1). Therefore the effect of flow behaviour is extremely important to this highly selective collapse process in causing damage. The suppression of flow-generated cavitation has been attributed primarily to higher viscoelastic stresses built up near the nose of a blunt body². Similarly, it may be expected that a proper application of the polymer additives may introduce higher stresses in the stagnation region which would lead to the following consequences. First, for a bubble travelling along the stagnation line, a higher external pressure would cause the bubble to collapse faster, and the chance for the cavity to be carried towards the surface is less. Second, for bubbles travelling along paths close to the stagnation line, higher stresses in the stagnation zone cause them to be repelled away from this region to take either path *A* or *B* shown in Fig. 1. A cavity that takes path *A* would be somewhat against the flow and quickly collapse in the liquid, causing practically no surface damage. On the other hand, a cavity following path *B* would be swept away downstream so that it would never come close enough to the surface to cause damage. Consequently, a reduction in flow-cavitation erosion may be expected. Experiments studying polymer effects on cavitation collapse and erosion in a flow system would be of some interest, and efforts along this line are now under way at this laboratory.

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Leaf cultivar influences composition and ripening of apples

POMOLOGICAL literature contains two reports of the influence of grafted scions on the size, colour and ripening season of apples borne on the stock portions of topworked trees^{1,2}. The experiment reported here, initiated as part of a larger study of apple ripening control mechanisms, confirms those reports.

Buds from a Lodi apple tree were grafted into branches of several Golden Delicious apple trees. When the Lodi scions bore fruit, half the scion branches were defoliated in early July, one month before the normal Lodi ripening season. Thereafter, these Lodi fruits received synthates translocated from Golden Delicious leaves. In late July, gas-sampling tubes (8 mm in diameter $\times$ 40 mm) were secured with grafting wax to the intact calyx ends of these Lodi fruits; the open end of each tube was fitted with a serum cap. Similar tubes were also secured to Lodi fruits borne on the other scion branches which still had Lodi leaves attached. The atmosphere inside the gas-sampling

located a few side branches below the Lodi graft unions. A similar procedure was followed in mid-September for limbs bearing Golden Delicious fruits.

Lodi fruits were judged to be ripe when the ethylene in the gas-sample tubes reached 10 p.p.m. or higher; or when, in a few cases, the fruits abscised or split open. Golden Delicious fruits, which ripen with lower levels of ethylene, were judged to be ripe when ethylene in the gas-sample tubes was 4.5 p.p.m. or higher. The data (Fig. 1) indicate that Lodi and Golden Delicious apples fed with synthates translocated from Golden Delicious leaves ripened 5–7 d earlier than comparable apples fed with synthates translocated from Lodi leaves. Although the source of the synthates influenced the time of fruit ripening, the source of the synthates did not seem to influence the level of ethylene in the apples once they began to ripen on the tree.

During the Golden Delicious ripening season, red stripes appeared on fruits fed with Lodi leaves. An analysis of skin composition indicated that, in comparison with similar fruit fed with Golden Delicious leaves, fruit fed with Lodi leaves contained higher levels of anthocyanin and flavonols, but similar levels of soluble carbohydrates, starch and flavanols (Table 1).

Table 1 Skin composition of Golden Delicious apples from girdled limbs fed with synthates from Golden Delicious or Lodi leaves

Leaves	Soluble carbohydrate (mg cm ⁻²) (4)	Starch (mg cm ⁻²) (4)	Anthocyanin (nmol cm ⁻²) (5)	Flavonols (nmol cm ⁻²) (6)	Flavonols as monomers (μ mol cm ⁻²) (5)
Golden Delicious	3.0	0.69	0.84	47.9	0.95
Lodi	2.9	0.75	4.09	101.0	0.90

Reference numbers in parentheses.

tubes equilibrated with apple internal atmospheres. A 1-cc syringe was used on alternate days to withdraw gas samples from the tubes and ethylene present in the samples was determined as described previously³. The ethylene analyses indicated the Lodi apples fed with synthates translocated from Golden Delicious leaves ripened slightly earlier than Lodi apples fed with synthates translocated from their own leaves.

In the second season, half of the Lodi-fruited branches were girdled in early July to prevent translocation of synthates from Golden Delicious leaves. On the same date, all the leaves were removed from the remainder of the fruited Lodi limbs. Lodi fruits on defoliated limbs received synthates translocated from Golden Delicious leaves. To ensure comparable accumulation of Golden Delicious leaf synthates in Lodi apples on the defoliated scions, bark girdles were

In summary, our analytical study verified orchard observations made in 1880 and 1927 that the cultivar of apple leaves may influence the composition and the time of ripening of apple fruits.

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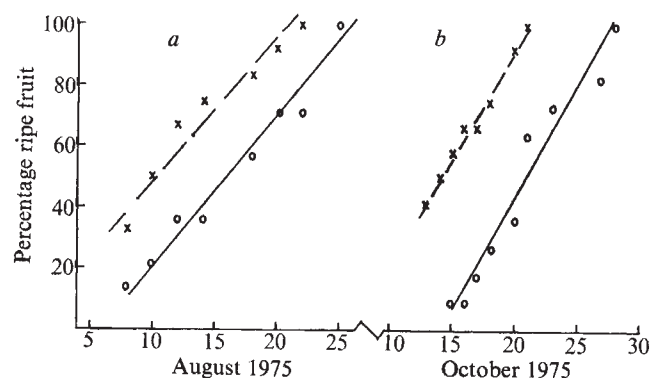
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Fig. 1 Ripening of *a*, Lodi and *b*, Golden Delicious apples borne on limbs fed with synthates from Golden Delicious ($\times$) or from Lodi ($\circ$) leaves. In each of the four treatments there was a total of 16 fruits on 6–10 trees.



Watering converts a CAM plant to daytime CO₂ uptake

THREE different photosynthetic options have been identified in plants^{1,2}: (1) most plants have the reductive pentose phosphate or C₃ pathway, where CO₂ is incorporated into ribulose-1,5-diphosphate (RuDP) to yield two molecules of 3-phosphoglyceric acid, a three-carbon compound; (2) the C₄ mode, where the first photosynthetic products are four-carbon dicarboxylic acids like oxaloacetate and malate formed following CO₂ incorporation into phosphoenolpyruvate (PEP); and (3) crassulacean acid metabolism (CAM), found in many succulent plants growing in arid regions. In the last, stomatal opening and net CO₂ uptake occur at night, CO₂ being incorporated by way of PEP carboxylase into organic acids. The tissue acidity decreases as the organic acids are decarboxylated

during the day, when the internally released CO_2 is prevented from leaving by the closed stomata. The water vapour concentration difference between the tissue and ambient air is less at night, and thus the night-time stomatal opening of CAM plants leads to overall water conservation. For example, the water lost per CO_2 fixed averages about sixfold higher for C_4 plants and tenfold higher for C_3 ones than for CAM plants in natural conditions². The net daily CO_2 uptake by CAM plants is less than for C_3 or C_4 plants, so CAM plants tend to be relatively slow growing.

We investigated the results of giving CAM plants an adequate water supply, thereby obviating the need for water conservation and show that this leads to their conversion to daytime CO_2 uptake. We used *Agave deserti* Engelm. (Agavaceae) plants, which are common in the sandy soils of the Colorado, Mojave and Sonoran deserts³, and experimented on plants maintained in a glasshouse so that the watering regime could be controlled. Gas exchange was measured with a Siemens null-point closed-circuit flow system^{4,5} with a Cambridge Systems EG&G 880 dew-point hygrometer for measuring water vapour concentrations and a Beckman 315A infrared gas analyser for CO_2 concentrations. At about weekly intervals, the distal end of an attached leaf representing about 100 cm^2 of surface area was sealed into the leaf chamber and maintained under natural illumination for a determination of gas exchange over 24 h. The resistance for water vapour diffusion from a leaf (R_{wv}) equals the concentration of water vapour within a leaf (assumed to be the saturation value at the leaf surface temperature) minus the water vapour concentration in the leaf chamber, divided by the measured rate of water vapour loss per unit leaf area. To measure leaf acidity^{6,7} approximately 4 g of leaf tissue were removed with a cork borer, ground in 100 ml of distilled water, boiled for 5 min, and then filtered. Two 40-ml aliquots were titrated to an endpoint of pH 6.4 using 0.011 N NaOH. For determination of PEP and RuDP carboxylase activities, each gram of freshly removed leaf tissue was ground in 5 ml of 0.2 M HEPES buffer (pH 8.0), 1 mM Na-EDTA, 1 mM dithiothreitol, and 40 mg ml^{-1} polyvinyl pyrrolidone; enzyme activities were assayed by following the conversion of $\text{KH}^{14}\text{CO}_3$ into stable products⁸. Soil water potential (Ψ_{soil}) was measured daily with a Wescor HR 33 dew-point microvoltmeter and PT 51-05 soil thermocouple psychrometers.

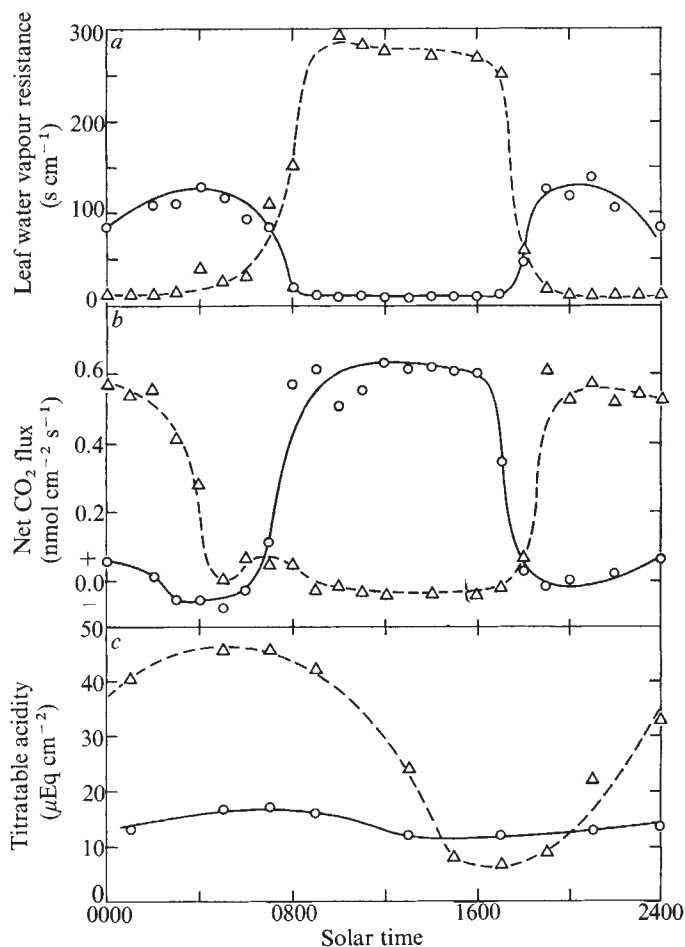
Four plants were potted in the field when no natural stomatal opening was occurring ($\Psi_{\text{soil}} < -90$ bar, where 1 bar = 10^5 Pa). Ψ_{soil} was then raised to -0.1 ± 0.1 bar, which induced night-time stomatal opening. After two weeks in a wet soil, CAM-type stomatal movements (Fig. 1a), a night-time CO_2 influx (Fig. 1b), and diurnal variations in the tissue acid level (Fig. 1c) were clearly evident. After four more weeks with a wet soil, the stomata remained open in the morning; the net CO_2 influx was $0.24 \text{ nmol cm}^{-2} \text{ s}^{-1}$ at 0900 and did not cease until 1130. After a total of 12 weeks with a Ψ_{soil} maintained at -0.1 ± 0.1 bar by daily watering, the night-time stomatal opening was replaced by a daytime opening, characteristic of C_3 and C_4 plants (Fig. 1a). Net CO_2 uptake (97% of the daily total) now occurred during illumination (Fig. 1b) and there was little diurnal change in tissue acidity (Fig. 1c). Allowing the soil to dry for 3 weeks caused the plants to revert to a more-or-less normal CAM mode with night-time stomatal opening and daytime closing.

When the plants were exhibiting photosynthesis primarily during the daylight hours and there was an apparent lack of the circadian fluctuation of organic acids, some form of either the C_3 or C_4 photosynthetic pathway was presumably being followed. The vascular tissue of *A. deserti* seemed to have a bundle sheath containing chloroplasts, but the cells were not as conspicuous as for most C_4 plants. Measurements of PEP carboxylase and RuDP carboxylase indicated a ratio of the activities similar to that observed for C_4 and CAM plants¹. For samples taken at 1200 from a plant exhibiting daytime stomatal opening, the PEP carboxylase activity was $1.01 \text{ } \mu\text{mol CO}_2 \text{ fixed per g wet weight per min}$ compared with 0.080 for RuDP

carboxylase. In the CAM mode (at 0000) there was also high PEP carboxylase activity relative to the RuDP carboxylase activity (0.80 as against $0.078 \text{ } \mu\text{mol per g wet weight per min}$). Whether the CO_2 assimilation in the light involves primarily PEP or RuDP carboxylase remains to be determined. The CO_2 compensation value measured at 1200 during daytime CO_2 uptake for plants at 17°C in saturating light conditions and 21% O_2 was relatively high (an average of $88 \text{ } \mu\text{l l}^{-1}$ for three experiments). This is not characteristic of C_4 plants (CO_2 compensation point generally less than $10 \text{ } \mu\text{l l}^{-1}$ (ref. 9)), but is similar to CO_2 compensation points for C_3 plants.

The maximum R_{wv} for *A. deserti* was twofold greater in the CAM mode ($\sim 300 \text{ s cm}^{-1}$, Fig. 1a) than for the daytime CO_2 uptake, indicating a tighter closing of the stomata when water conservation is more critical. The daytime stomatal opening did not produce a much greater maximum rate of photosynthesis than the CAM night-time opening (0.63 compared with $0.57 \text{ nmol cm}^{-2} \text{ s}^{-1}$, Fig. 1b). These maximum rates are two- to sixfold lower than those for conventional C_3 and C_4 plants², primarily because of the rather high resistance for the gas phase part of the pathway for *A. deserti* (minimum R_{wv} of about

Fig. 1 Conversion from crassulacean acid metabolism (CAM, Δ) to daytime CO_2 uptake ($\circ$) after watering *Agave deserti*. Gas exchange data were obtained in identical conditions using attached leaves 2 weeks (CAM) and 12 weeks after raising Ψ_{soil} from < -90 bar to -0.1 bar. Acidity was determined for leaves on a companion plant showing the same stomatal movements. Air in the leaf chamber was maintained at $17.0 \pm 0.4^\circ\text{C}$ and $5.0 \pm 0.1 \text{ } \mu\text{g H}_2\text{O cm}^{-2}$. Illumination was provided by natural sunlight (supplemented with a xenon arc lamp during brief cloudy periods) and controlled using neutral density screens so that it was representative for a cloudless winter day (for example, January 1, 1976). Illumination had a maximum of 80 klx at 1200 with values of 10 klx at 0730 and 1645, as determined using a Spectra FC 200-TV-B meter. Data are expressed on the basis of total area (both sides) for these succulent leaves.



7 s cm⁻¹ for both modes). This succulent arid-region plant would thus still grow relatively slowly if switched to the daytime mode of photosynthesis. For the CAM mode, the net CO₂ fixation was 18.3 μmol cm⁻² for 24 h (Fig. 1b), whereas it was 21.0 μmol cm⁻² for daytime CO₂ uptake. These values of net daily photosynthesis were obtained in conditions of wintertime illumination. The relative advantage of predominantly daytime photosynthesis would be greater for the longer days (a longer period for daytime CO₂ uptake) and shorter nights (less time for the CAM night-time CO₂ influx) that would occur at other times of the year. The time course of the increase in titratable acidity closely followed the net accumulation of CO₂ in the leaf during the night-time period in the CAM mode and 2 μEq of acidity were produced per μmol CO₂ taken up as expected (Fig. 1b and c).

When water is readily available, strictly adhering to the water-conserving CAM mode may no longer be beneficial to a CAM plant. At least some supplemental daytime stomatal opening and CO₂ uptake would become advantageous. Indeed, many CAM plants show a net CO₂ uptake during the day when not under water stress; such daytime CO₂ uptake increased as water became more available for *Bryophyllum daigremontianum*¹⁰, *Agave americana*¹¹, and *Dudleya farinosa*¹². The shift to daytime photosynthesis was only partial, however, as in all cases the night-time stomatal opening and net CO₂ influx characteristic of CAM plants still occurred. In different experiments, Osmond *et al.*¹³ showed that subjecting *Kalanchoe daigremontiana* to 9 weeks of drought caused the carbon isotope discrimination ratio to shift from a value close to that for C₃ plants towards that expected if all CO₂ uptake took place at night in a CAM mode. For *A. deserti*, it seems that watering can cause an essentially complete switch from CAM to daytime photosynthesis (Fig. 1). Such switching did not lead to greater net daily photosynthesis for winter days, but greater photosynthetic productivity would be predicted for the longer daylight periods occurring at other times of the year. Thus, the ability to shift modes of photosynthesis when water is not a limiting factor could be a distinct advantage to this plant.

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Memory phases in *Drosophila*

MEMORY in many organisms can be disrupted by anaesthesia or electroconvulsive shock applied shortly after training. Later, if left undisturbed, the memory becomes immune to these agents. This suggests that learned information is stored by the brain in more than one form^{1–4}. A population of *Drosophila melanogaster* can be trained to avoid an odorant by presenting the odour in combination with electric shock. When tested later without shock, the flies avoid this odorant specifically (ref. 5

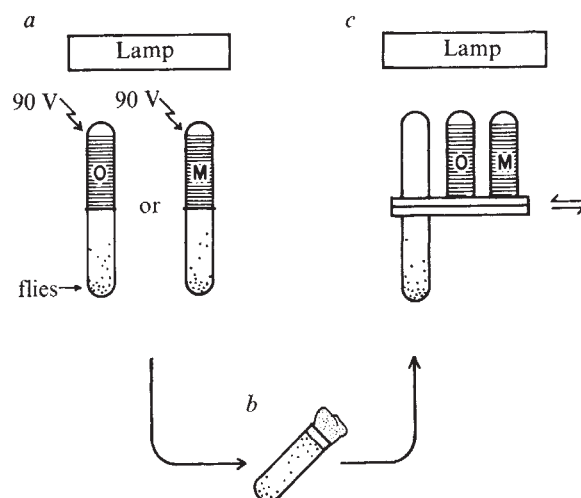


Fig. 1 Procedure for measuring memory retention. *a*, Training: a population of 30–50 flies was trained in two horizontal plastic tubes, joined open end to open end (in a behavioural counter-current device⁵). The light attracted the flies from a start tube, containing 1 μl of a chemical odorant (either 3-octanol (O) or 4-methyl-cyclohexanol (M)) spread on an electrified grid (60 Hz, 90 V a.c.)⁶. After 60 s, the flies were shaken back into the start tube and another run was begun. This cycle was repeated ten times. By the tenth training cycle fewer than 20% of flies entered the grid tube. *b*, Waiting: flies were left undisturbed in dim illumination at room temperature (22 °C), in a perforated plastic tube closed with a polyurethane foam plug. *c*, Testing: the sliding counter-current apparatus⁵ allowed the start tube, with flies, to be shifted opposite any of several illuminated tubes. Selective odour avoidance of the flies was measured by testing them alternately with two (non-electrified) grids, one spread with O, the other with M. In each test, the flies were shifted opposite a tube containing the grid and odorant and given 15 s to run, after which the flies remaining in the start tube were counted. Before each test, flies were allowed to run to a perforated rest tube for 45 s. The order of odorants was reversed in half the experiments.

and unpublished results of Y.D.). By anaesthetising the flies briefly with cold at various times between training and testing, we have found two memory components in *Drosophila*.

D. melanogaster of the Canton-special wild-type strain were used. Culture methods, experimental conditions, chemical odorants and the apparatus for training and testing were as described before⁵, except that, to enhance memory retention, we used a more intensive training procedure consisting of ten consecutive exposures to one odorant coupled with shock (Fig. 1a). Between training and testing, flies were left undisturbed in ventilated plastic tubes (Fig. 1b). They were tested, as before⁵, by allowing them to run sequentially into two new grid tubes (non-electrified), each containing one of the odorants above (Fig. 1c). The index of learning used was the fraction of the population which avoided the odorant associated with shock during their training, minus the fraction which avoided the novel (control) odorant. Each experiment was run in reciprocal halves; two groups of flies, each trained to avoid one of the odorants, were both tested in the same apparatus to compensate for asymmetries in the apparatus or in the flies' intrinsic reaction to the odorants. The learning index, Λ , for a complete experiment was the average of the indices for the two halves. Learning indices are reported as mean $\pm$ s.e. of mean for four experiments. With the training procedure described above, selective avoidance was present immediately after training, $\Lambda = 0.25 \pm 0.02$, and remained high; when tested 45 min after training $\Lambda = 0.34 \pm 0.06$.

Brief cooling induces rapid and reversible anaesthesia in *Drosophila*. Flies to be treated were transferred to a glass test tube (18 $\times$ 150 mm), shaken to the bottom, and the tube was placed in a bath of moving water at 4 °C. Within 15 s the flies stopped climbing, fell over and became immobile. After a

further 60 s they were transferred back to perforated plastic tubes at room temperature (22 °C). Two minutes later they began to walk, and locomotion appeared normal by 7 min.

We tried to disrupt the flies' memory by applying this cold treatment at various times before and after training, and in each case measured their retention 45 min after training (Fig. 2). Flies anaesthetised before training (at -10 or -5 min) perform as well as untreated flies, indicating that the cooling *per se* does not interfere with subsequent neural function, sensory perception or capacity to learn and remember. Flies anaesthetised 0 or 5 min after training, on the other hand, showed almost no selective avoidance when tested at 45 min. Apparently cold-induced anaesthesia can disrupt recently acquired memory in *Drosophila*. From 10 to 30 min after training, the memory became progressively more resistant to anaesthesia. This time-dependent increase suggests the onset of a second memory phase. The following experiments examine alternatives to this interpretation.

Populations of flies were trained as above, immediately anaesthetised, maintained at room temperature and tested at 5, 15, 30, 45 or 60 min. In all cases they showed little retention, with values of Λ less than 0.10. This indicates that in *Drosophila* cold-induced anaesthesia after training either eradicates learned information or permanently interferes with its normal expression. The amnesic effect of cold anaesthesia is not an artefact of the time chosen for testing.

Memory-disruption experiments are complicated by the possibility that the disruptive agent, applied shortly after training, itself acts as a reinforcement, causing gratuitous learning which cancels the effect of previous training⁶. In our case, the learning task is aversive, and anaesthesia decreases avoidance of the shock-associated odorant. Therefore, the anaesthesia cannot act as a negative reinforcement. Positive reinforcement effects were excluded by a separate control: populations of flies were cold-anaesthetised in the presence of either 3-octanol or 4-methylcyclohexanol, at the concentrations used above. When tested later they showed the same slight aversion to both odorants as did naive control flies.

Time-dependent sensitivity of memory to cold anaesthesia has also been demonstrated using the original paradigm of Quinn *et al.*⁵, which contains an internal control of sensitisation. Flies were trained by alternate exposure to two odorants, one of which was associated with shock. Testing was as above. Cold anaesthesia disrupted memory if applied 5 min after

training ($\Lambda = 0.05 \pm 0.04$), but not if applied at 30 min ($\Lambda = 0.18 \pm 0.04$) (unpublished results of Y.D.).

The extent to which the mechanisms underlying memory storage are shared among species is unknown. Nevertheless, analogous techniques can be used to resolve memory phases in creatures ranging from dipterans to primates. Among the insects, multiple memory components have been shown in cockroaches by use of lesions⁷, and in honey bees by use of anaesthesia and electroconvulsive shock⁸. The demonstration of two forms of memory in *Drosophila* may be useful because of the suitability of this organism for behaviour-genetic analyses⁹. At present, work on memory phases is limited to phenomenology, and fundamental questions remain unanswered. Short and long term memory storage could proceed by different physiological processes, or the second phase could represent merely a saturation or intensification of the first. Information may proceed serially through short term to consolidated memory, or the two forms may have parallel inputs. These questions could be studied if mutations affecting different memory components were obtained. In this respect, it is interesting that a single-gene mutant deficient in learning has been isolated recently¹⁰.

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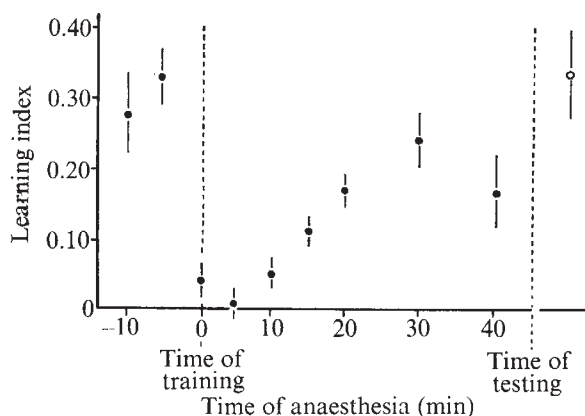
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Fig. 2 Effect of cold anaesthesia on memory. Populations of 30-50 flies were trained and tested 45 min later as described in Fig. 1. Populations were anaesthetised at the times shown, by cooling them for 60 s at 4 °C. (The decreased learning performance of flies anaesthetised at 40 min may be due to incomplete recovery by testing time.) The abscissa shows time between training and cold anaesthesia. Negative times indicate anaesthesia before training; positive values indicate anaesthesia after training and testing. The point shown after testing (hollow circle) represents unanaesthetised control flies. The ordinate shows Λ , the selective avoidance due to learning.



Maintenance of fluid volume in the starfish water vascular system

LOCOMOTION in the starfish *Asterias forbesi* involves many tube feet, each functioning independently as a hydrostatic skeleton; the circular muscles of the ampulla acting antagonistically to the longitudinal muscles of the tube foot itself through the constant volume of fluid contained in the ampulla-foot unit¹. The fluid for each tube foot comes from the water vascular system to which each foot is connected through its own lateral canal. The water vascular system in *Asterias* consists of three interconnecting series of canals: (1) radial canals running the length of each arm; (2) a circular canal running around the gut at the base of the arms, and (3) the stone canal which runs from the radial canal up to the aboral ("dorsal") surface, terminating in the madreporite. The madreporite, an orange disk, is porous and associated with several sets of cilia. For some time it has been presumed, and is still presented or indicated in some textbooks, that the fluid contained within the starfish water vascular system is pumped by ciliary activity through the

madreporite into the canal system, although it has been pointed out that no experimental evidence supports this assumption². Ionically, the fluid within the water vascular system is nearly identical to the external seawater with the exception of internal K^+ , which is present in a concentration up to 60% higher than that of the seawater^{3,4}. On the basis of this difference, it was suggested that K^+ accumulation by the water vascular system is responsible for water uptake by this system, either by creating a slight osmotic gradient within the tube feet or by direct movement of water with hydrated K^+ ions, as opposed to direct uptake of seawater through the madreporite⁵. We have investigated the generation of the fluid in the water vascular system more thoroughly by determining the osmotic and ionic characteristics of the fluid within the tube feet and the ionic transport characteristics of the isolated tube foot epithelium.

We used *A. forbesi* collected in Jamestown, Rhode Island and maintained for up to 1 week before use in tanks of artificial seawater at 7 °C. Fluid from the water vascular system was obtained from an intact starfish by placing it on its aboral surface and tying off individual tube feet close to their base with silk thread. The foot was then isolated from the animal, washed quickly in distilled water, blotted dry and cut open. The released fluid was taken up into a micropipette. Na^+ and K^+ concentrations in the tube foot fluid were determined by flame photometry, and Cl^- was measured by a modified microelectrometric titration technique⁶, and the total osmolality of the fluid was obtained with a Clifton nanolitre osmometer. These determinations are summarised in Table 1. The Na^+ concentration of the tube foot fluid was identical to that of the external medium, while internal Cl^- was slightly but significantly higher than environmental Cl^- . In agreement with earlier measurements on other echinoderms^{3,4}, the K^+ concentration of the tube foot fluid from *A. forbesi* was considerably higher than that of the external seawater. In addition, the tube foot fluid was distinctly hyperosmotic to the surrounding seawater, by up to 22 mosM. The perivisceral (coelomic) fluid was osmotically and ionically (Na^+ , K^+ and Cl^- ion concentrations) identical with the external seawater.

To investigate whether or not the tube foot epithelium itself is in part responsible for K^+ secretion, individual tube feet were cut off at the base, filled with fresh seawater and tied off with silk thread. Thus initially identical ion concentrations were present on both sides of the isolated tube foot epithelium. The tube foot-sac preparation was allowed to equilibrate in aerated seawater for 90 min. Analysis then showed that, as in the intact tube foot, the K^+ concentration of the fluid in the isolated tube foot was higher than that of the surrounding medium. The internal K^+ concentration was 16.8 mM, while that in the external seawater was 9.5 mM. The secretion of K^+ by the isolated tube foot epithelium could be inhibited by addition of CN^- (1×10^{-4} M) to the external medium. The isolated tube foot could also be set up as a tubular system and perfused⁷; in this manner unidirectional K^+ fluxes, using ^{42}K (New England Nuclear), could be measured. Unidirectional K^+

influx was 4.2×10^{-9} mol cm^{-2} min^{-1} and unidirectional K^+ efflux was 3.4×10^{-9} mol cm^{-2} min^{-1} .

Connection of the tube feet with the outside medium through the canals of the water vascular system and the madreporite was investigated by equilibrating intact starfish for 24 h in seawater with ^{14}C -polyethylene glycol (New England Nuclear); molecular weight 4,000. Samples of fluid were then removed from the tube feet as before and counted in a liquid scintillation counter. Negligible amounts of label were found inside the tube feet, indicating that no direct connection exists between the fluid in the tube feet and the outside medium across the madreporite, an observation consistent with studies performed with dyes⁸.

None of these observations supports the assumption that the fluid in the tube feet of the starfish is simply seawater pumped into the water vascular system through the madreporite by the action of cilia. The fluid in the tube feet, as well as that in the connecting water vascular system, is higher in K^+ and Cl^- , and is hyperosmotic with respect to the external seawater. This hyperosmotic condition is probably brought about by the active secretion of K^+ , with Cl^- following passively, across the tube foot epithelium. Other epithelial structures in addition to the tube foot epithelium itself in the starfish may contribute to total K^+ secretion into the water vascular system, for example, the ampulla epithelium. The generation of a slightly hyperosmotic condition in the tube foot lumen by solute secretion would create a driving force for water across the tube foot epithelium into the tube foot lumen. This water provides the fluid through which the circular muscles of the ampulla and the longitudinal muscles of the foot are antagonised. In addition, water uptake in this fashion could make up for water losses through ultrafiltration across contracted tube feet and through damaged tube feet.

The lack of any direct connection of the madreporite with the more distal portions of the water vascular system in the starfish does not rule out any exchange whatsoever between different parts of the water vascular system and the external seawater through the madreporite. The water vascular system in some echinoderms is probably connected entirely to the outside environment through the madreporite. Water movements which do occur through the madreporite in asteroids may serve either a sensory role or could serve to equalise pressure differentials⁸ between the water vascular system and the outside environment.

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Table 1 Osmotic and ionic characteristics of fluid from intact starfish tube feet and of surrounding seawater

Ion concentration (mmol kg^{-1})	Seawater	Tube feet fluid
Na^+	469 ± 2.9 (17)	471.4 ± 2.6 (17)
K^+	9.5 ± 0.8 (21)	17.3 ± 1.8 (21)
Cl^-	543.3 ± 2.6 (19)	551.7 ± 3.1 (19)
Total osmolality mosmol l^{-1}	1,095 ± 3.3 (25)	1,117 ± 3.8 (25)

Figures are mean ± s.e.m., with number of determinations in parentheses.

Evolution of responses to relative homozygosity

ACCORDING to the theory of kin selection first elaborated by Hamilton^{1,2}, all individual behaviour is selected to maximise the increment to inclusive fitness $\Delta W_i + \Sigma \Delta W_j r_{ij}$, where ΔW_i is the effect of an act on the fitness of the actor, ΔW_j is the effect of the same act on the fitness of a conspecific, and r_{ij} is the coefficient of relationship between the

actor and the conspecific. Acts that lower an individual's direct fitness can thus be favoured by natural selection if, by conferring benefits on relatives of the actor, they cause a positive increment to the actor's inclusive fitness^{3,4}. Since the average r_{ij} between an individual and the targets of its behaviour varies because of fluctuations in local population structure, an individual is most likely to secure a positive increment if its behaviour is conditioned on as accurate an estimate as possible of the particular local distribution of r_{ij} . I describe here a mechanism by which this estimate could be improved on the basis of information derived from the relative homozygosity of the individual's own genotype, in species with certain kinds of life histories.

A geographically viscous, sexually reproducing population is locally inbred, reflecting the fact that the parents of a typical individual are more highly related to each other than are two adults taken from the population at large⁵. Since relative homozygosity correlates with the extent of inbreeding, an individual that is homozygous at an unusually high proportion of its population's polymorphic loci is almost certain to be one whose ancestors for several preceding generations were closely related. In a viscous population, neighbouring individuals tend to share largely overlapping sets of ancestors. Thus given any mating system, characteristic pattern of dispersal, and level of genetic polymorphism, an individual more (or less) homozygous than its population's recent average expects a larger (or smaller) than average coefficient of relationship to conspecific near neighbours⁶. In many species these neighbours are likely to be its principal ecological competitors.

Particular developmental and behavioural responses to homozygosity could be favoured by natural selection, if the relationship between individual fitness and some limiting resource can be described by an increasing function that becomes concave downwards above a level of use commonly reached by members of the population. The summed fitness of two individuals who divide a given resource may then exceed the fitness either of them would attain by taking all of that resource. Given sufficiently high r_{ij} and a sufficiently large shareable resource, each individual is selected to take less than all of that resource and so attain less than the direct fitness it might. An unusually homozygous individual may, on average, increase its inclusive fitness by being relatively small and reproductively efficient. A very heterozygous conspecific may be selected to exhibit greater competitiveness over the shareable limiting resource because its inclusive fitness is less likely to be increased by any benefits (positive ΔW_i) it bestows on neighbours.

The logic of this argument has been tested in a series of numerical experiments. The model species is monoecious, semelparous and synchronously breeding. Each site on a 24×24 stepping-stone array accommodates, at most, one individual, who may be more or less 'competitive'. This phenotypic variance in competitiveness has two selective effects. First, the risk of juvenile mortality is a positive linear function of the competitiveness (and the number) of an individual's immediate neighbours. Second, more competitive individuals draw resources from less competitive neighbours, and fertility is an increasing sigmoid function of resource accrued. Competitiveness is controlled by two alleles at each of four independently assorting loci. One allele at each locus is dominant for maximum competitiveness; the other is recessive for reduced competitiveness. Phenotypic effects of the four loci are multiplicative. Symmetrical binomial probabilities govern the dispersal of male gametes and of zygotes, which experience average migrations of slightly more than one step and slightly less than one step, respectively.

Each experiment simulated one hundred generations of selection and reproduction. The recessive allele was strongly favoured at rates of self-fertilisation ranging between zero and 0.975. Gene frequencies changed very little during cor-

responding control runs in which all genotypes produced the same intermediate level of competitiveness. Individual fertility in experimental runs was observed to correlate positively with competitiveness. Thus, the success of the allele that responded to homozygosity by lowering the competitiveness of its phenotype could have been due only to benefits it conferred on replica copies occurring in neighbours.

Predictions derived from this model have been tested on published hybridisation experiments from a number of species. Embryos *in utero* stand in competition for a bounded set of resources. Relatively homozygous embryos, with high coefficients of relationship to each other, should compete less strongly than relatively heterozygous embryos. Not only should they be smaller than the heterozygotes, they should also be less variable at birth if competition tends to increase the variance of resource accrual. Where crosses are made between completely inbred lines there exists among F_1 littermates no genotypic variance to which their phenotypic variance might be attributed. Taketomi and Matsuo reported a large and well controlled hybridisation experiment with mice⁷, where the coefficient of variation of weight at 10 d of age was significantly larger for F_1 hybrid than for inbred litters ($P < 0.01$). This difference is unlikely to have been caused by some special incompatibility between inbred females and their hybrid embryos, because backcross litters on inbred females were less variable than backcross litters on F_1 females ($P < 0.01$).

For another series of hybridisation studies on mice, there is a strong and significant positive correlation between the hybrid litters' heterosis for birth weight and their 'heterosis' for variability⁸. This supports the assumption that intrauterine competition for resources tends to increase the variance of birth weight.

Barley is a predominantly self-fertilising species with very low rates of pollen and seed dispersal. In several hybrid populations synthesised from parents of diverse origin, yields per unit area and average homozygosities both increased during early generations^{9,10}, although barley is strongly heterotic for individual yield¹¹. Available evidence does not permit an unequivocal rejection of the possibility that selection produced late generation plants that were as large as early generation plants and even higher yielding. But since late generation plants were very homozygous, it seems likely that they were also smaller and individually less fit than their more heterozygous ancestors. Their greater yields per unit area are then assumed to reflect higher densities and greater efficiency in the utilisation of resources such as light. The response of individual yield to plant spacing in barley is smoothly asymptotic¹², so selection in a viscous population would seem to favour a response to homozygosity like that modelled in the numerical experiments.

In these barley populations, however, the seeds used to initiate each generation were a large and thoroughly randomised sample produced the previous generation. Therefore differentiated neighbourhoods could not actually develop. Close relatives would seldom have received the benefits dispensed by uncompetitive individuals. In such unusually heterogeneous and chaotically arrayed populations, selection should favour those homozygous genotypes that lead to relatively unreduced competitiveness. Electrophoretic surveys on one of these populations revealed an apparent narrowing of the difference between single-locus homozygous and heterozygous fitnesses within the first twenty-eight generations¹³, in spite of a marked increase of gametic phase disequilibrium¹⁰. During the same series of generations, highly significant increases in the rate of outcrossing also occurred¹⁴. Both of these changes can be interpreted as evolutionary responses to a situation in which direct fitness and inclusive fitness have become nearly synonymous.

The accuracy of an estimate of average genetic relationship to near neighbours that is derived from homozygosity must depend on the number of loci entering into the estimate, and on the independence with which those loci assort. Traits whose expression depends heavily on the amount of fitness-limiting resources accrued are thus expected to show low additive genetic variance, relatively high non-additive genetic variance and strong heterosis, and this pattern is widely observed^{15,16}. Much of the inbreeding depression seen in normally outbred species must result from the expression of truly deleterious recessive alleles. But such alleles should be relatively rare in species that inbreed to any considerable extent; particularly in such species, the loss of direct fitness that accompanies inbreeding may result in part from mechanisms favoured by natural selection.

Published observations seem to satisfy many predictions derived from the hypothesis, but plausible alternative explanations can be advanced for most and none constitutes a clear critical test. An important and largely unexamined general implication of the model is that particular features of the mating and genetic systems of species to which it applies, should seem designed to facilitate the proposed responses to relative homozygosity.

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Non-random inactivation of X chromosome in the rat yolk sac

THE Lyon hypothesis postulates that the mammalian female is a natural mosaic for clones of cells with either the maternally derived X chromosome (X^m) or the paternally derived one (X^p) which is randomly inactivated early in development^{1,2}. We have presented evidence for the dominance of the inactive X^p in extraembryonic regions of 7.5- and 8.5-d mouse embryos heterozygous for Cattanach's translocation³ in which the two X chromosomes could be readily identified⁴. Though we presumed that this might not be an exceptional phenomenon restricted to mice bearing this X-autosome translocation, this has been difficult to confirm because of the lack of a system suitable for experiments. Here we report further cytological evidence that the inactive X is predominantly paternal in the yolk sac of the laboratory rat.

We used X^L-X^s dimorphism in the laboratory rat⁵; the X chromosome is telocentric (X^L) in F344 and ACI strains, whereas it is subtelocentric (X^s) in the WKA strain bred at

our laboratory (I. Hayata, unpublished). X^LX^s heterozygotes were obtained from the cross $ACI(X^LX^L) \times WKA(X^sY)$ and $WKA(X^sX^s) \times F344(X^LY)$. The X chromosome transmitted from the father is X^s in the former cross, whereas it is X^L in the latter. Embryos were recovered from the decidual swelling at 10.5 d gestation when the yolk sac had enveloped the amnion, and the allantois had fused with the chorion. Mosaic composition was studied only in the embryo proper, and in the yolk sac, by means of a technique essentially similar to the one described elsewhere^{6,7}.

Distinction between X^s and X^L was unequivocal and an asynchronously replicating X was identified as the palest red element in the great majority of metaphase spreads with banded chromosomes (Fig. 1). It should be mentioned, however, that the short arm of the isocyclic X^s failed to show up in occasional black and white photographs. Cytogenetic examination was carried out in 8 and 7 embryos from the cross $X^LX^L \times X^LY$ and $X^LX^L \times X^sY$, respectively. In the former 8 embryos the percentage of cells with the X^p allocyclic ranged from 82.1% to 97.8% with an average of 94.7% in the yolk sac, whereas it ranged from 46.7% to 71.4% with an average of 55.3% in the embryo proper. The difference between the two was highly significant ($P < 0.001$). The results were similar in the latter 7 embryos; the average for the yolk sac, although slightly lower

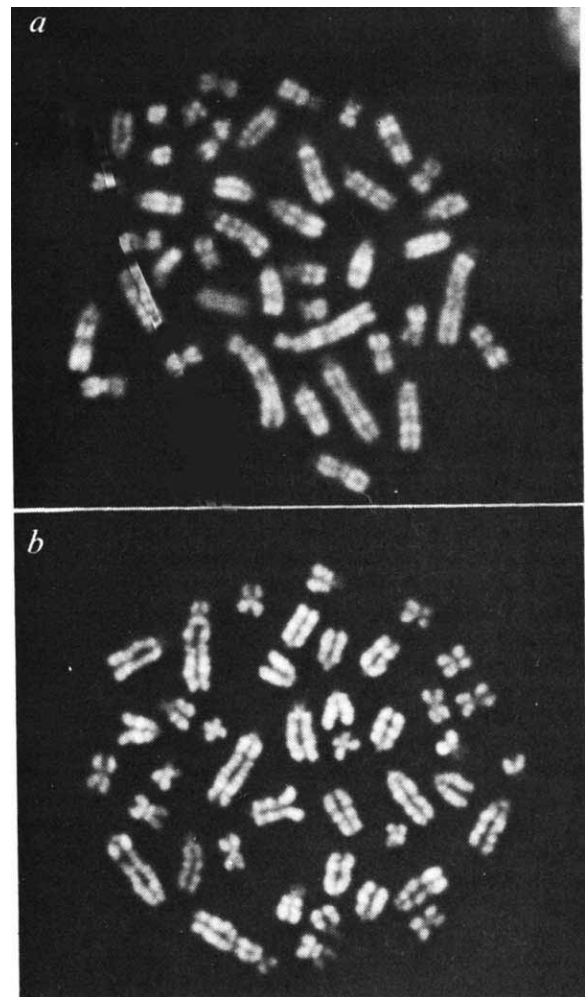


Fig. 1 BUdR-AO differential staining of the allocyclic X chromosome in cells of a 10.5-d-old X^LX^s embryo from the cross $X^LX^L \times X^sY$. The allocyclic X which fluoresced faintly is maternal (X^L) in *a*, whereas it is paternal (X^s) in *b*. Embryos were taken out of the uterine cavity, incubated at 37 °C for 10 h in Eagle's medium supplemented with foetal calf serum (20%) and bromodeoxyuridine ($100 \mu\text{g ml}^{-1}$). Colcemid ($1 \mu\text{g ml}^{-1}$) was added 1 h before collection. Air-dried slides were prepared according to a modified method of Wroblewska and Dyban⁶ as described by Takagi and Oshimura⁷, and stained with acridine orange (0.5 mg ml^{-1}) in 0.15 M Sørensen's phosphate buffer (pH 7.0).

Table 1 Parental influence on X-inactivation mosaic composition in 10.5-d-old female rat embryos

Embryo	Area examined	No. of cells examined	No. of cells with allocyclic X ^p (%)
(1) X^mX^m × X^mY			
1	Y	46	45 (97.8)
	E	67	42 (62.7)
2	Y	169	155 (91.7)
	E	152	97 (63.8)
3	Y	67	55 (82.1)
	E	135	63 (46.7)
4	Y	152	148 (97.3)
	E	84	53 (63.1)
5	Y	242	233 (96.3)
	E	173	86 (49.7)
6	Y	302	288 (95.4)
	E	136	67 (49.3)
7	Y	82	79 (96.3)
	E	35	25 (71.4)
8	Y	53	51 (96.2)
	E	26	14 (53.8)
Total	Y	1,113	1,054 (94.7)
	E	808	447 (55.3)
(2) X^mX^m × X^mY			
1	Y	162	146 (90.1)
	E	103	71 (68.9)
2	Y	165	145 (87.9)
	E	275	162 (58.9)
3	Y	93	84 (90.3)
	E	124	55 (44.4)
4	Y	201	182 (90.5)
	E	179	95 (53.1)
5	Y	219	199 (90.9)
	E	124	69 (55.6)
6	Y	121	109 (90.1)
	E	107	63 (58.9)
7	Y	160	154 (96.3)
	E	140	83 (59.3)
Total	Y	1,121	1,019 (90.9)
	E	1,052	598 (56.8)

Y, Yolk sac; E, embryonic body.

compared with that in the former embryos, was not statistically significant ($0.1 < P < 0.2$). It seemed that there was more variation in mosaic composition among embryos themselves than yolk sacs, as indicated by a significantly higher standard deviation ($P < 0.05$). It should also be mentioned that there was no correlation in the proportions of the two cell types between these structures within an embryo ($r = 0.33$, $0.2 < P < 0.3$). Thus the present study substantiated the previous observation made in the mouse heterozygous for Cattanach's translocation. Another aspect in common with the mouse embryo was a slight excess of cells with the X^p inactive in the embryo proper. But the source of departure from the equal representation expected on random basis is not known.

We have already discussed elsewhere³ possible ways to bring about the extreme mosaicism and its potential significance. A salient feature may be mentioned in connection with the recent findings. There are at least three possible ways of explaining the apparently preferential inactivation of the paternal X chromosome: (1) reactivation of the inactive X^m with simultaneous inactivation of the active X^p; (2) selective growth of cells with the X^m active after random inactivation; (3) preferential inactivation of the X^p. Takagi (in preparation), by means of a double labelling method with ³H-thymidine and 5-bromodeoxyuridine, could exclude the first possibility in mouse embryos. Which of the remaining two is more likely? Isozyme activities of glucose-6-phosphate dehydrogenase in various organs from mules strongly suggest *in vivo* selection for cells with the horse X active in certain organs. Extra-embryonic regions in mouse and rat might also be under strict selective pressure which favours the cell having the X^m active. Our recent observation (N.W. and N.T., unpublished) in 3.5-d mouse embryos, on the contrary, suggests that the

Table 2 Comparison of average percentages and standard deviations of cells with an allocyclic X^p in the yolk sac and embryo proper

Cross type	Yolk sac	Embryo proper
X ^m X ^m × X ^m Y	94.1* ± 4.9	57.5 ± 8.3
X ^m X ^m × X ^m Y	90.9* ± 2.4†	57.0 ± 6.9
Combined	92.6* ± 4.2†	57.3 ± 7.3

* $P < 0.001$.† $P < 0.05$ (compared with the embryo proper).

initial event may not be random, in that the X^p was allocyclic in more than 90% of informative metaphases. Resolution of this problem will require fuller cytological studies of embryos in early development.

The result reported here implies that the overwhelming excess of the X^p inactive in the yolk sac and probably in some other extraembryonic tissues may hold at least in the family *Muridae*. Further studies will disclose whether it is an evolutionary specialisation in a small group of species, or a widespread phenomenon among eutherian mammals. In either case, it seems highly unlikely that the specific pattern of inactivation has been retained without any advantage. Cells having the active X^m may somehow be advantageous for the interaction between mother and embryo after implantation. We may assume that the majority of primordial germ cells, arising in the yolk sac, would possibly have the active X^m. If this is the case, it is tempting to consider the extreme mosaic composition in the yolk sac as a mechanism controlling X chromosome activity in female germ cells.

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Parallel distribution of sister chromatid exchanges and chromosome aberrations

THE role of chromatid exchange in the origin of chromosome aberrations has been a subject of research since the chromatid exchange hypothesis of chromosome aberrations was proposed¹. Which of the breakage-reunion hypothesis² and the exchange hypothesis better explains the origin of chromosome aberrations is still disputed^{3,4}. The part played by DNA repair in aberration and exchange formation has been identified^{5,6}, and there is much evidence for non-random distribution of chromosome aberrations, especially in the heterochromatic regions^{7,8}. We have shown previously by autoradiography, that sister chromatid exchanges (SCEs) induced by 7,12-dimethylbenz(a)anthracene (DMBA) are frequently associated with aberrations⁹. Newly introduced 5-bromodeoxyuridine (BUdR)—33258 Hoechst methods for SCEs^{9,10} have made it possible to reanalyse these problems in more detail and with considerable accuracy. The results presented here indicate that two different hydrocarbon carcinogens, DMBA and 7,8,12-trimethylbenz(a)-

anthracene (TMBA), induce aberrations localised in the same chromosomal regions of the rat bone marrow cells and that the targets for chromosome aberrations and for SCE are identical; in other words, both phenomena are related.

Two of the largest chromosomes (Nos 1 and 2) of the rat have extremely vulnerable regions to DMBA, especially when the carcinogen is administered shortly before cell collection, probably because of late DNA replication in these regions⁸. These vulnerable sites were therefore used to study the relationship between chromosome aberration and SCE. Male Long-Evans rats (30 d old) were used and were injected intravenously with a lipid emulsion of DMBA or TMBA (Upjohn) at 50 mg per kg body weight, 6 h before killing. Colchicine (0.3 mg) was administered intraperitoneally 1 h before killing and chromosome specimens were prepared from femur bone marrow by the standard air-drying method and stained with Giemsa. Chromosome aberrations observed consisted of gaps and breaks. Gaps were defined by a clear achromatic lesion with continuity, and breaks by displacement of the distal segment. Both ends of a break, or a gap from the centromere projected on the chromosome axis, were measured as accurately as possible in photographic prints and expressed by the percentage in the length of these chromosome arms. Distribution curves were plotted of the relative location of 100 breaks and gaps along both chromosomes. For SCE, an *in vitro* system was used, because *in vivo* BUdR-labelling of rat bone marrow cells was unsuccessful. The bone marrow cells from femur were suspended in 5 ml of thymidine-free F-12 medium containing 15% foetal calf serum and $2 \mu\text{g ml}^{-1}$ of BUdR and were cultured for 26 h, or two rounds of the cell cycle⁸. DMBA or TMBA (5×10^{-4} mM) was dissolved in 0.1 ml of dimethylsulphoxide, and $0.2 \mu\text{g ml}^{-1}$ of colchicine were added to the medium 6 and 2 h respectively before collecting the cells. Chromosome specimens were prepared and differential staining of sister chromatids was carried out using the modified 33258 Hoechst-Giemsa method¹¹.

Fig. 1 Distribution of DMBA-induced chromosome aberrations (a) and sister chromatid exchanges (b) along the long arms of No. 1 and No. 2 chromosomes. The graphs are a combination of results from several samples. Numbers on the abscissa represent percentage distance from the centromere; 0, centromere; 100, telomere. The ordinate indicates the frequency per 100 aberrations in each chromosome. Six hours after DMBA (50 mg kg^{-1} , intravenously), 23.4% of metaphase bone marrow cells had aberrations. The mean number of aberrations per cell was 0.27; 13.7% were in No. 1; 17.9% in No. 2; and 68.4% in the remaining chromosomes. Of the aberrations 14% were breaks as defined by chromatid displacement and the rest were gaps. On the other hand, 289 SCEs were found in 26 metaphases (11.5 per cell) 6 h after addition of DMBA (5×10^{-4} mM) *in vitro*; 9.7% were in No. 1; 11.7% in No. 2; and 78.5% in the remaining chromosomes.

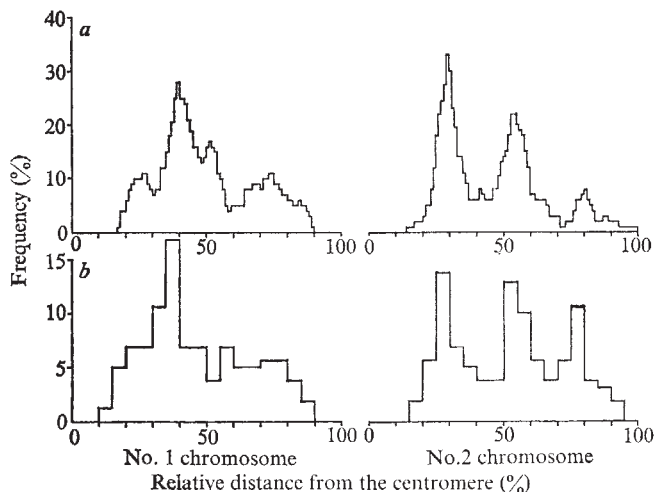
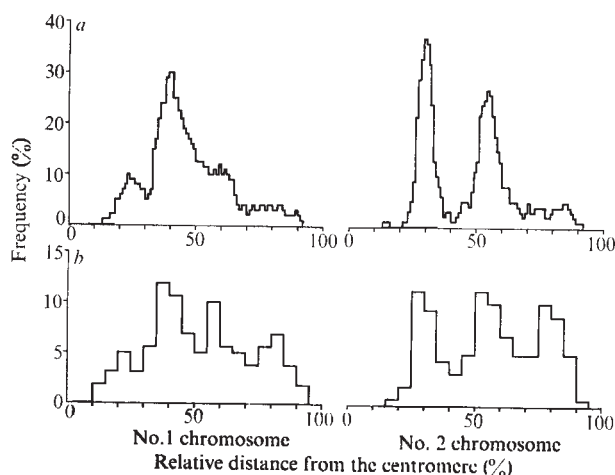


Fig. 2 Distribution of TMBA-induced aberrations (a) and sister chromatid exchanges (b) along the long arms of No. 1 and No. 2 chromosomes. At 6 h, 17.7% of metaphase bone marrow cells revealed aberrations. The mean aberration per cell was 0.20; 18.6% were in No. 1; 22.9% in No. 2; and 58.6% in the remaining chromosomes. Of the aberrations 14.5% were breaks and the rest were gaps. 253 sister chromatid exchanges were found in 26 metaphase cells (9.7 per cell) 6 h after addition of TMBA (5×10^{-4} mM) *in vitro*; 10.2% were in No. 1; 9.0% in No. 2; and 76.7% in the remaining chromosomes.

One hundred SCEs in the long arms of both chromosomes were located in photographic prints by measuring the relative distance from the centromere. The distribution curves of SCEs were made by plotting the relative site of each exchange along the chromosome axis divided into 20 segments.

The DMBA- and TMBA-induced breaks and gaps were shown to distribute along the chromosomes non-randomly and reproducibly (Figs 1 and 2). The No. 1 chromosome had a susceptible site 40% of the distance along the long arm. In the No. 2 chromosome, there were three target sites for breaks and gaps; they were about 30, 55, and 80% along the chromosome from the centromere, although the last peak was not evident in the animals referred to in Fig. 1 treated with DMBA. SCEs were, however, also distributed non-randomly. The distribution curves of exchanges in No. 1 chromosome were similar to those for aberrations. There were also three susceptible regions for exchanges in the No. 2 chromosome. They coincided completely with the target sites for aberrations although the third susceptible region was far more susceptible to exchanges than to aberrations. Some SCEs were associated with aberrations (Fig. 3).

These results indicate that aberrations and SCEs are correlated. From this correlation, it is possible that aberrations represent incomplete SCEs that were not repaired.

Fig. 3 Sister chromatid exchanges in the susceptible regions of No. 1 chromosome (a) and No. 2 chromosome (b, c and d). b and d, Exchanges are incomplete, but are associated with a gap.



Whether SCEs comprise 25% of the total chromosome lesions as expected from Revell's hypothesis¹ is unknown from the present studies. Because late DNA replication in and the late vulnerability of these susceptible regions to DMBA have been suggested⁸, it is possible that DNA replication is important in the induction and non-random distribution of these changes, although the part played by other factors such as repetitious DNA, intranuclear distribution of carcinogens, and chromosome association in the interphase nuclei⁷ cannot be excluded. These non-random actions of chemical carcinogens need further studies in relation to carcinogenesis, because they parallel carcinogenicity of the chemicals^{5,12} and the chemical carcinogens with a completely different chemical structure such as 4-nitroquinoline-1-oxide and *N*-nitroso-*N*-butylurea also induce the same genetic effects (unpublished data).

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Communication through fimbriae during conjugation in a fungus

LONG fine hairs, termed fimbriae¹ or pili², have been observed in many bacteria and in fungi^{3–5}. Bacterial sex pili transfer chromosomal and plasmid DNA during conjugation⁶. This paper describes evidence that the fimbriae of the smut fungus *Ustilago violacea* also transfer sex-specific molecules between the two conjugants.

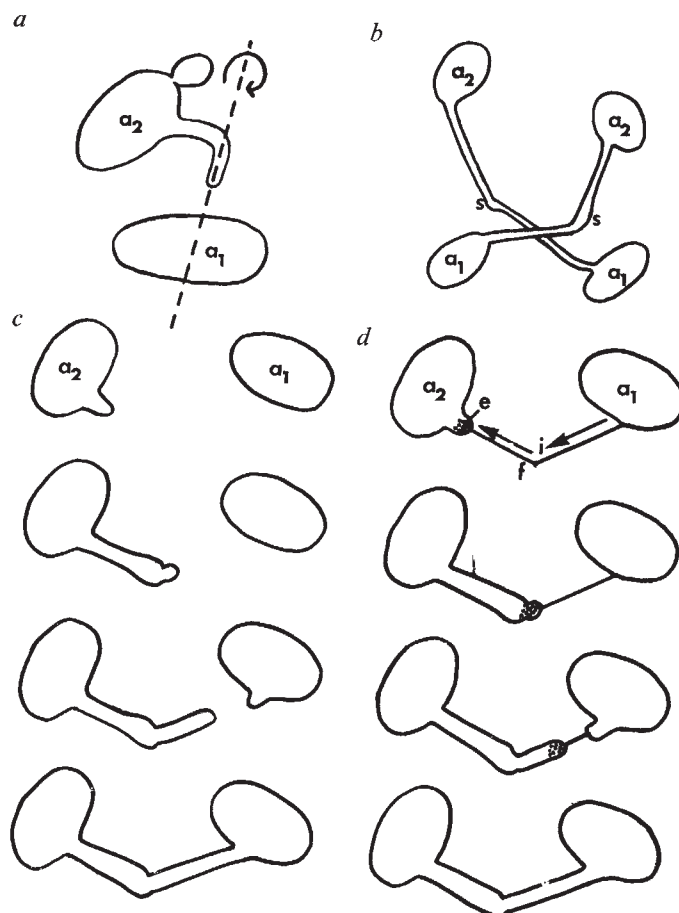
The cells of this fungus may mate while they are touching (contact mating) or at distances of up to 20 μ m on water agar (distant mating)^{5,7}. During distant mating a tube grows from the *a*₂ mating type to the other cell (*a*₁ mating type; Fig. 1). Fimbriae up to 15 μ m long are found on both mating types and are therefore long enough to account for distant mating, if it is assumed that the fimbriae of the two mating cells may attach end-on. The evidence that such fimbrial contacts between cells are formed and that they are essential for conjugation is as follows.

The behaviour of the cells, as observed under the light microscope, provides very strong evidence for the existence of an invisible connecting fibre at distances of more than 5 μ m apart. Such cells floated together in water suspensions, rotating in unison and maintaining a constant maximum distance apart. The axis of rotation indicated that the cells were connected by a fibre that was laterally flexible but torsionally rigid, and was attached to the cell surface at a discrete site. They therefore rotated very similarly to two balloons fixed together with thin wire. The

connection was fragile and could be broken by pressure on the cover slip. When water suspensions of cells were made carefully, such attached cells were found in mating cultures, but not in pure cultures or in mixed cultures of opposite mating type kept in conditions unsuitable for mating. Fimbriae are the only structures on the cell wall which could act as these invisible fibres.

The conjugation tube in cells mating at a distance seemed to grow along the line of the fimbrial connection. On nine occasions to date cells were observed similar to those in Fig. 1*a*, in which one mating partner produced a conjugation peg that had grown part way towards the other cell. The cells remained connected by an invisible thread and in every case their rotation as a pair clearly showed that the invisible fibre connected the tip of the conjugation tube to the other cell. The distance covered by the fibre varied

Fig. 1 *a*, Pair of mating cells attached by an 'invisible' fibre as observed under the light microscope in a water suspension. Dotted line represents axis of rotation of the pair of cells. *b*, Route taken by conjugation tubes from a pair of *a*₂ cells to two *a*₁ cells. Note that the tubes do not necessarily contact the nearest partner, and that they may swell before changing direction. *c*, Pair of cells during distant mating, showing the development of the conjugation tube from the *a*₂ cell, the swelling at the point where the direction changes and the growth of a small peg on the *a*₁ cell opposite the tube. *d*, Interpretation of the stages in *c*. The cells are considered to be connected by end-to-end joining of fimbriae *f*, from each partner. An inducer (*i*) is transported along this fimbrial connection from the *a*₁ cell to the *a*₂ cell. The inducer stimulates local production of wall-softening enzyme (*e*), at the *a*₂ terminus of the fimbrium, causing development of a peg. Further transport of inducer along the fimbriae results in continued growth of the tube along this path. As the growing tube nears the other cell transport of enzyme or of an inducer from *a*₂ may occur in a reverse direction causing some tube development on the *a*₁ cell.



from 1 to 5 μm ; probably longer fimbrial connections tend to break in water suspension.

During distant mating the tube frequently did not grow directly across to the other cell but curved or turned at a sharp angle. Frequently the tip of the tube swelled before resuming growth in a new direction (Fig. 1b-d). The tube sometimes passed by a nearby cell of opposite mating type to reach one further away (Fig. 1b). The nearer cell later mated with another cell. These observations are difficult to explain if chemical diffusion is the cause of tube growth, but are readily explained if the tube follows the line of a previously established fimbrial connection.

Observations of several closely related species of *Ustilago* have established that only fimbriated strains can conjugate. Thus Kniep⁹ showed that strains of *U. scabiosae*, *U. cardui*, *U. vinosa*, *U. utriculosae* and *U. violacea* will conjugate with each other. Modern strains of these species that have become afimbriate have also lost the ability to conjugate, whereas strains that still produce fimbriae have full mating activity.

In an earlier report⁸ it was shown that fimbriation and conjugation both have the same maximum temperature^{2,5}, the same response to temperature shifts, the same response to enzyme treatments, and are both blocked before translation by high temperatures. The cells still grow in conditions which limit both conjugation and fimbriation.

Electron microscopy of thin section and freeze-etched preparations demonstrated that fibres of similar appearance to fimbriae connect cells that are very close together^{5,7,8}. These fibres were also found on the ends of the conjugation tubes^{5,7}. Unfortunately, the fragility of the connection in distantly mating cells has so far frustrated attempts to demonstrate it by shadow casting.

Although these results establish that fimbriae are essential for conjugation, their exact function is not yet known. It is possible that there are both common fimbriae and sex fimbriae as in bacteria, since one or two fimbriae may differ from the others in having a terminal knob⁵, just as frequently occurs with the sex pili of bacteria⁶, and since only one or two conjugation tubes are produced per haploid cell, although there are hundreds of fimbriae on most cells.

The growth of the conjugation tube along the line of the fimbrial connection suggests that this fibre may be used to provide chemical communication between the cells⁵. It is suggested that 'inducer' molecules are transmitted along the fimbrial connection mainly from the a_1 cell to the a_2 cell, and that these molecules stimulate production of a wall-softening enzyme locally near the a_2 terminus of the fimbrial connection. This would cause bulging of the cell wall at the fimbrial terminus and initiate development of the conjugation tube. Further transmission of inducer together with continued growth in the a_2 cell would cause the tube to grow at its tip along the line of the fimbrium. If the connection between the two fimbriae is a poor one, inducer may accumulate at the junction resulting in swelling of the tip of the conjugation tube before growth along the fimbrium is resumed. The tendency of the a_1 cell to produce a conjugation peg as the conjugation tube approaches (Fig. 1c and d) may be due to movement of the enzyme itself along the fimbrium, or to the slow transport of inducer molecules from a_2 to a_1 .

The inducer is perhaps most likely to be an enzyme subunit or coeffector, but it could also be a regulatory protein, messenger RNA or perhaps even DNA. The ability of fimbriae to transmit such large molecules was doubted for a while but has received strong support from recent work with bacteria⁶, including the decisive experiments of Ou and Anderson¹⁰ who showed that *E. coli* cells could complete conjugation and undergo recombination while remaining separated by distances of up to 3 μm and connected

only by an 'invisible' thread. The similarity between the ultrastructural features of conjugation in bacteria and in *Ustilago* is very striking in view of the differences in nuclear organisation. It remains to be seen to what extent this similarity extends to the biochemical, genetical and regulatory levels.

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Induction of "natural killer" cells by BCG

THE past decade has witnessed numerous attempts to suppress tumour growth by the administration of immune stimulants. Notable successes have been achieved, particularly following the intrasplenic injection of Bacille Calmette-Guérin (BCG) which has been observed to cause the regression of local tumour nodules in both experimental animals^{1,2} and man³⁻⁵. Although there is some controversy as to the mode of action of BCG, it has become widely accepted that its anti-tumour effects are principally mediated through host macrophages, "activated" by the immunostimulant⁶⁻⁸. Observations we have recently made in studies of the peritoneal exudates of BCG-infected mice suggest an alternative possibility: that tumour destruction is caused by a non-macrophage population of cytotoxic cells which is induced by BCG immunisation.

Young adult (6-8 weeks) C57BL/6 mice were immunised intraperitoneally with 10^7 viable BCG organisms (Pasteur Strain, Trudeau Institute, New York). Peritoneal exudates were harvested at frequent intervals thereafter and the lytic activity of washed cells assessed against a variety of ^{51}Cr -labelled target cells using a 4-h microcytotoxicity assay⁹. Exudates from unimmunised animals were not cytotoxic under these assay conditions. In contrast, peritoneal cells obtained 2-15 d after

Table 1 Lytic activity of murine peritoneal exudates

Treatment used to induce exudate	% Specific cytotoxicity	
	P815-X2*	EL-4 (G)-†
BCG	21	15
Thioglycollate	0	2.9
Proteose peptone	0	2.2
None (normal PEC)	0	0.5

*A methylcholanthrene-induced mastocytoma of DBA/2 origin, maintained in ascites form.

†A methylcholanthrene-induced thymoma of C57BL/6 origin, maintained in ascites form.

Young adult C57BL/6 mice were immunised intraperitoneally with 10^7 viable BCG organisms (Pasteur strain, Trudeau Institute, New York), with 2 ml 3% (w/v) thioglycollate medium or with 1.5 ml 10% (w/v) proteose peptone. Peritoneal exudates were collected 3 d later and lytic activity of 1.5×10^6 cells assayed against 10^4 ^{51}Cr targets. A 4-h microcytotoxicity assay was performed in RPMI 1640 containing 10% foetal calf serum. Specific cytotoxicity was calculated relative to target cells in the absence of any effector cells. The standard deviation of quadruplicate cultures was $\pm 2\%$ of the mean. We have also observed extensive lysis of Chang (human liver cell line) cells by BCG exudates and, to a much reduced extent, by exudates induced by thioglycollate and proteose peptone (data not shown).

Table 2 Characteristics of the peritoneal exudate killer cell

Expt	Treatment of effector cells	% Specific cytotoxicity
(1)	None	18
	Adherent to tissue culture dish	7.7
	Non-adherent	37
(2)	None	15
	Carbonyl iron	25
(3)	None	22
	C	17
	Anti- θ +C	17
(4)	None	17
	C	17
	Anti-Fab+C	19

Peritoneal exudates were collected from C57BL/6 mice 3–6 d after immunisation with 10^7 viable BCG organisms. The following fractionation procedures were performed in attempts to identify the cytotoxic cell. (1) Exudates were centrifuged (300g, 3 min) on to a tissue culture dish (Falcon Plastics) and were then incubated for 40 min at 37 °C. Non-adherent cells were decanted, adherent cells removed with 5 mM EDTA in ice-cold Ca^{2+} -free HEPES buffer. (2) Carbonyl iron depletion was carried out by method of Lundgren¹⁴. (3) The anti- θ serum used was an AKR anti-C3H thymus previously described¹⁵. Normal rabbit serum was used as the source of complement (C). (4) A rabbit anti-mouse Fab serum was a gift from Dr Keith James, University of Edinburgh. Rabbit serum was again used as a complement source. The cytolytic activity of the fractionated populations was in each case assessed in a 4-h assay using 5×10^6 effector cells and 10^4 ^{51}Cr -labelled P815 target cells.

immunisation invariably caused target cell destruction. While there was considerable animal to animal variation in the amount of cytotoxicity exhibited, peak lytic activity was observed in exudate populations harvested 4–8 d after immunisation. The decline in reactivity was gradual, and some lytic activity was noted in populations harvested up to 3 weeks after BCG administration.

Several features of the lysis observed deserve comment: (1) the amount of lysis occurring in a 4-h period was directly proportional to the number of exudate cells added to the cultures (range used: 2×10^5 – 2×10^6 exudate cells per 10^4 target cells); (2) lysis, over a 3-h incubation period, was a linear function of time; and (3) C57BL/6-derived peritoneal exudates were capable of lysing syngeneic EL-4 thymoma targets as well as allogeneic P815 mastocytoma targets. Data from a typical experiment are shown in Table 1. The lytic effect noted was not confined to the C57BL/6 mouse; similar cytotoxicity has been observed with BCG induced exudates in the DBA/2 mouse. Interestingly, while thioglycollate and proteose peptone induced more copious, macrophage-rich, peritoneal exudates than did BCG, such cell populations were only minimally lytic (Table 1).

Attempts were made to characterise the nature of the cytotoxic cell present in BCG-induced exudates. The cytotoxic cell did not adhere to tissue culture plates (Table 2) nor was it

phagocytic. Depletion of phagocytes by treatment of 2×10^7 peritoneal exudate cells with 200 mg carbonyl iron for 40 min at 37 °C, followed by application of magnetic field, removed 60% of the total cells. There was a concomitant increase in the lytic activity of the residual population (Table 2). It thus seems extremely unlikely that the cytotoxic cell in the exudates is a macrophage.

The lytic activity of BCG-induced peritoneal exudates was not affected by treatment with an AKR-antiserum directed against θ -alloantigen in the presence of a source of rabbit complement (Table 2). Identical treatment removed 91% of the T cell-mediated cytotoxic activity of spleen populations obtained from C57BL/6 mice immunised with P815 (DBA/2) alloantigen (data not shown). We conclude therefore that the killer cell in BCG exudates is not a thymus-derived lymphocyte.

Cells bearing surface immunoglobulin were quantitated by the use of a fluoresceinated goat IgG with anti-mouse Fab activity. Treatment of exudates with a rabbit antiserum to the Fab portion of mouse IgG in the presence of rabbit complement diminished the number of fluorescent cells from 12% to 2.4%. This treatment was without effect on the lytic activity of the exudates (Table 2). We conclude that the killer cell lacks surface immunoglobulin.

Soluble immune complexes were without effect on cytotoxicity by BCG-induced exudate cells under conditions which readily inhibited antibody-dependent K cell-mediated cytotoxicity (Table 3). This suggests that the BCG exudate cytolytic cell is not dependent on an Fc receptor for its activity. Furthermore, although K cells are notably trypsin resistant¹⁰, cytotoxicity by BCG exudates was inhibited by trypsinisation of the effector cells before the assay (Table 3).

The effector cell present in BCG-induced peritoneal exudates therefore lacks: (1) the adherent and phagocytic characteristics of macrophages, (2) the surface characteristics of both T and B lymphocytes, and (3) the Fc-dependency and trypsin resistance of the K cell. By these criteria the cell is comparable to the "natural killer" cell described by Herberman *et al.*^{11,12} and by Kiessling *et al.*^{10,13}.

To further this comparison, we investigated whether the exudate cell possessed an additional feature which has been reported to be peculiar to the natural cytotoxic cell: namely, a marked lability at 37 °C in culture¹¹.

Peritoneal exudate cells harvested 5 d after BCG infection were cultured at 37 °C at a concentration of 5×10^6 cells ml^{-1} in RPMI 1640 containing 10% foetal calf serum. After incubation for varying periods of time at 37 °C, the cells were held at 4 °C until a total time of 4 h had elapsed. At this time ^{51}Cr -labelled P815 cells were added, the cultures transferred to an incubator at 37 °C and the lysis of target cells evaluated 4 h later. As can be seen from Table 4, the lytic activity of exudate cells was extremely labile, a 50% reduction in lytic activity being observed within 7 h (3 h incubation + 4 h assay) at 37 °C. In contrast, T cell-mediated cytotoxic activity of alloimmune

Table 3 Inhibition of cytotoxicity by immune complexes or trypsinisation of effector cells

Effector cells	Target cells	Immune complex inhibition			Trypsin sensitivity		
		Antibody control	Complex	Inhibition (%)	Control	Trypsin	Inhibition (%)
Alloimmune spleen	P815	16	17	0	8.7	0.2	98
Normal spleen	Antibody coated CRBCs	14	3.6	74	4.5	5.0	0
BCG exudate	P815	20	23	0	23	12	48

Alloimmune spleen cells were obtained by culturing normal C57BL/6 mouse spleen cells with DBA/2 stimulating cells for 4 d in the condition described by Grottni *et al.*¹⁶. Normal spleen cells were from C57BL/6 mice, and peritoneal exudate cells were obtained from the same strain 4 d following BCG inoculation. Both alloimmune spleen and BCG-induced peritoneal exudates were assayed against 10^4 ^{51}Cr -P815 target cells, the former at a 5:1, and the latter at a 50:1 effector–target cell ratio. Antibody-dependent cytotoxic (K cell) activity was measured by culturing 10^6 normal spleen cells with 5×10^4 ^{51}Cr -labelled chicken erythrocytes (CRBCs) which had been coated with a 10^4 dilution of a mouse anti-CRBC serum. Soluble immune complexes were prepared by incubating a rabbit anti-DNP antiserum ($40 \mu\text{g ml}^{-1}$) with DNP-BSA ($4 \mu\text{g ml}^{-1}$) for 1 h at 37 °C. A 0.05-ml volume of this mixture was then added to 0.1 ml of effector cells and incubated 30 min at 37 °C, followed by addition of target cells in a 0.05-ml volume. Trypsin treatment of effector cells was carried out for 45 min at 37 °C using a cell concentration of 2×10^7 cells ml^{-1} , and either 0.25% trypsin (Sigma) or medium alone. Cells were washed and adjusted for viable cell recovery before assay. All cultures were in the presence of target cells for 4 h at 37 °C.

spleen (or peritoneal exudate cells) did not fall during identical culture conditions (Table 4 and unpublished observations).

In summary we have observed a cytotoxic cell population in the peritoneal exudates of mice 2–15 d after infection with viable BCG organisms. The cell population lacks those characteristics that are associated with B and T lymphocytes, macrophages and K cells. The BCG exudate cytotoxic cell shows a marked lability to temperature that the directly cytotoxic T cell does not. Additionally, in agreement with Kiessling *et al.*¹⁰, it shows a susceptibility to trypsin treatment similar to the cytotoxic T cell, but in contrast to cytotoxic K cells. The exudate killer cell we describe here thus seems similar, if not identical, to the natural cytotoxic cell previously described^{10–13}.

It is interesting to note therefore that Herberman's studies¹¹ have shown that the mouse strain used here (C57BL/6), and specifically its peritoneal exudate, has a very low incidence of natural cytotoxic cells. This is presumably reflected in an inability to detect lytic activity in our assay system using normal exudate populations. It is not yet clear which of several alternatives is responsible for the vastly augmented activity seen in exudates after BCG administration. The principal possibilities seem to be: (1) that BCG induces a cytotoxic population *ab initio*; (2) that BCG causes the expansion of a population of cytotoxic cells which is initially present at an incidence below detection; (3) that BCG recruits into the peritoneal cavity cells which normally reside in other compartments or (4) that BCG "activates" an exudate cell population, rendering it cytotoxic.

Table 4 Lability of the cytotoxic cell

Cell	Time (h) 37 °C	Time (h) 4 °C	Specific cytolysis (%)	Inhibition (%)
BCG peritoneal exudate	0	4	27	0
	1	3	30	0
	2	2	21	21
	3	1	13	52
	4	0	6.4	76
Alloimmune spleen	0	4	68	0
	1	3	67	2
	2	2	69	0
	3	1	74	0
	4	0	78	0

Peritoneal exudates were harvested 5 d after immunisation of 6-week-old C57BL/6 mice with 10^7 viable BCG organisms. Allo-immunised spleens were obtained from C57BL/6 mice 10 d after immunisation with 3×10^7 P815 cells. Effector cells were incubated alone at 37 °C for various periods of time and then placed in an ice water bath at 4 °C until a total time of 4 h had elapsed. At this time 5×10^5 effector cells were incubated for 4 h at 37 °C with 10^4 ^{51}Cr -P815 cells and the degree of target cell destruction evaluated. Inhibition following culture at 37 °C was calculated relative to the activity of effector cells stored continuously at 4 °C.

Further investigations will clearly be needed to define the nature of the inductive process, to delineate the lineage of the cytolytic cell, and to see whether other bacterial products with anti-tumour reactivity (for example *C. parvum*; MER) also produce cytotoxic cells. It may not be coincidental that of the three agents we have used to induce peritoneal exudates only BCG substantially augments natural cytotoxicity and only BCG shows anti-tumour activity *in vivo*.

The induction of cytotoxic cells following BCG administration suggests to us that this population might be at least partially responsible for the tumour regression associated with BCG therapy. Furthermore, these observations imply that measurement of the natural cytotoxic cell population might prove to be a useful adjunct to studies aimed at optimising therapy with bacterial adjuvants.

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Spleen cells from *Schistosoma mansoni*-infected mice produce diffusible stimulator of eosinophilopoiesis *in vivo*

WE have previously demonstrated that a diffusible stimulator of eosinophilopoiesis is produced in mice after secondary injection with tetanus toxoid (TT)¹. Lymphoid cells from TT-primed animals are capable of producing a diffusible eosinophilopoietic stimulator in non-primed hosts on intraperitoneal TT injection². To establish that this is not a unique phenomenon, limited to this antigen, but rather that it is characteristic of antigen-induced eosinophilias and associated eosinophilopoietic responses, we have studied eosinophilopoiesis in a parasite-induced eosinophilia.

Mice with experimental schistosomiasis exhibit marked peripheral eosinophilia³, also observed in patients with this infection⁴. Spleen cells (SC) from *Schistosoma mansoni*-infected mice were tested for their ability to produce a diffusible stimulator of eosinophilopoiesis in response to an injection of a soluble schistosomal egg antigenic (SEA) preparation⁵.

Prospective SC donors were injected intraperitoneally with 125 *S. mansoni* cercariae 8–12 weeks before use; control SC donors were untreated. Peripheral eosinophil levels in infected mice remained at normal levels (<5%) until between weeks 7 and 8 after infection. They then increased to 7–14%, at which level they remained until the last animal was killed at week 12. Liver and lung sections from infected mice contained large eosinophil-containing granulomas surrounding schistosoma ova.

The quadrachamber diffusion assembly² was used to culture normal C57BL/6Ja bone marrow cells transfilter *in vivo* from SC from *S. mansoni*-infected, or normal, non-infected, isogenic mice. Chamber assemblies were implanted in the peritoneal cavities of non-infected C57BL/6Ja or B6D2F₁ (C57BL/6Ja × DBA/2) mice. One hour after implantation, host mice received an injection of 25 µg protein SEA intraperitoneally. Six days after implantation, chambers were recovered and assayed for total and differential cell counts (see Fig. 1).

After killing and assaying, significantly greater total and

Table 1 Quadrachamber diffusion assembly bone marrow cellularity 6 d after intraperitoneal implantation in normal C57BL/6Ja mice*

Spleen cell source†	Total cells	Chamber marrow cellularity†		Granulocytes (non-eosinophil) ($\times 10^{-6}$)	Eosinophils ($\times 10^{-6}$)	% Eosinophils Granulocytes
		% Granulocytes (non-eosinophil)	% Eosinophils			
Uninfected mice (13)§	0.77 $\pm$ 0.08	40.88 $\pm$ 1.91	2.95 $\pm$ 0.36	0.324 $\pm$ 0.040	0.022 $\pm$ 0.004	6.5 $\pm$ 0.6
<i>S. mansoni</i> -infected mice (13)	0.65 $\pm$ 0.07	35.70 $\pm$ 2.33	7.01 $\pm$ 0.59	0.232 $\pm$ 0.029	0.042 $\pm$ 0.004	17.0 $\pm$ 1.6
P value¶	NS	NS	<0.001	NS	<0.005	<0.001

* Data are summarised from two independent experiments.

† Mean $\pm$ s.e.m.‡ 10^7 cells from the source indicated. All host mice received 25 μ g protein-soluble (SEA) preparation intraperitoneally within 1 h after implant.

§ Number of chamber assemblies assayed.

¶ Test for significance determined by independent *t* test on data from paired chambers only.NS, Not significant ($P > 0.1$).

percentage eosinophil granulocyte counts were present in bone marrow populations transfilter from infected SC compared with those transfilter from non-infected SC (Table 1). Total eosinophils were 0.042×10^6 compared with 0.022×10^6 in controls ($P < 0.005$), representing a percentage eosinophil granulocytes of 7.01 and 2.95, respectively ($P < 0.001$). The difference in eosinophil levels was not attributable to differences in total cell counts, percentage granulocytes or total granulocytes, as no significant differences were seen in these parameters for the two bone marrow cell populations. Expression of eosinophils as a percentage of total granulocytes (%E/TG) gives a further indication that increases in eosinophil levels did not correlate with total granulocyte changes. In these studies, the %E/TG was 17.0% transfilter from infected SC compared with 6.5% across from non-infected SC, confirming the specificity of the stimulus for eosinophils.

These results indicate that spleen cells from schistosoma-infected mice are capable of producing a diffusible substance stimulatory for eosinophilopoiesis and are consistent with previous experiments that demonstrated the produc-

tion of a diffusible eosinophilopoietic factor by TT-primed lymphoid cells on secondary challenge.

Increased peripheral eosinophil levels are seen in experimental animals and man during the course of *S. mansoni* infection. It has been shown that eosinophil granulocytes are prime components in the immune response to schistosoma eggs⁶, and *in vitro* studies indicate that they may additionally be involved in immune-dependent destruction of schistosomula. It is therefore not surprising that an eosinophilopoietic factor would be produced in schistosomiasis.

Functional lymphoid cells are necessary for eosinophil response to antigens in which this has been tested^{8,9}. The lymphoid cells evidently produce the stimulator of eosinophilopoiesis *in vivo* as a part of their response to antigens which elicit eosinophilia and medullary eosinophilopoiesis, as in the case of TT and schistosomiasis. Murine or human lymphoid cells from schistosoma-infected individuals produce a lymphokine-termed eosinophil stimulation promotor (ESP) on exposure to SEA *in vitro*^{10,11}. ESP causes increased eosinophil migration in the *in vitro* assay conditions. Whether the eosinophilopoiesis stimulating factor is identical or distinct from ESP needs to be determined.

Further understanding of the processes regulating eosinophil production may assist our understanding of the reaction to parasitic infection, and the mechanisms of granulopoiesis.

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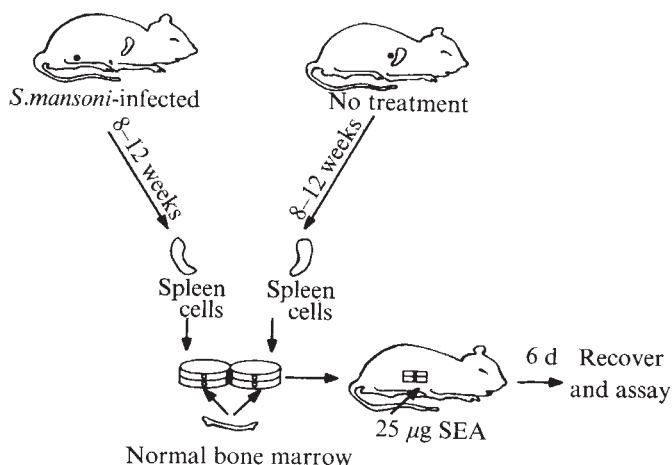
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Fig. 1 Quadrachamber diffusion assembly assay. SC donors received either 125 *S. mansoni* cercariae intraperitoneally or no treatment 8-12 weeks before quadrachamber diffusion assembly implantation. Chamber assemblies composed of two diffusion chamber doublets with 0.22 Millipore filters on both sides, and between upper and lower chambers, enable diffusion within doublets, and with the peritoneal fluid, but not directly between doublets. Upper chambers received 10^7 spleen cells from either the normal or schistosoma-infected donors in 0.1 ml suspending fluid (6 parts M199 : 2 parts buffer : 1 part plasma-EDTA). Lower chambers received 10^6 normal bone marrow cells in 0.1 ml suspending fluid. Chamber assemblies were implanted intraperitoneally in C57BL/6Ja or B6D2F₁ mice. Within 1 h after implantation, host mice received an intraperitoneal injection of 25 μ g SEA in 0.2 ml saline. Six days later chambers were recovered, shaken in 0.5% protease in 5% Ficoll in PBS, and individual bone marrow chamber contents were assayed for total and differential cell counts.



Novel approach to the treatment of hydatid disease

HYDATID disease is a cyclozoonotic infection of man and certain other mammals which results from the cystic intermediate stage of the cestodes *Echinococcus granulosus* and *E. multilocularis*. The growth of these two species of parasites is basically similar except that the latter undergoes tumour-like metastatic dissemination. The present knowledge about immunity to this disease is incomplete; however, recent reports from this laboratory¹⁻³ have defined certain basic aspects of the immune response to secondary echinococcosis. These studies have demonstrated the rapid *in vitro* lysis of protoscolices by the complement proteins when these are activated by surface immunoglobulins acquired *in vivo* by the larvae within the cysts. We also have evidence (unpublished data) that host serum proteins, including IgG and IgM immunoglobulins, are found within the cyst tissues of *E. multilocularis*, as previously demonstrated in *E. granulosus*⁴; Rau and Tanner⁵ have demonstrated that heat-inactivated serum from infected hosts is cytotoxic to the protoscolices of *E. multilocularis in vitro*. Based on these results, we now hypothesise that anti-*Echinococcus* antibodies in the serum of infected animals can "recognise" antigenic sites on the parasite but are unable to reach these sites in sufficient quantities under the natural conditions of the infection; given the opportunity of contact, these cytotoxic antibodies would subject the parasites to the deleterious effects of the immune response. We now present results which show that cysts of *E. granulosus* are killed when the fluid within these cysts is replaced with the fresh serum of infected animals and suggest this immunotherapy as a novel treatment of hydatid disease. Similar replacement of the fluid of *E. multilocularis* cysts also successfully killed the treated cysts; but the cyst mass continued to grow by surface budding.

In a series of experiments, sterile and fertile free peritoneal cysts of *E. granulosus* from infected A/J mice were transplanted into the peritoneal cavities of normal cotton rats (*Sigmondon hispidus*). Sixty days later, laparotomy was performed and the cysts treated as described in Table 1.

When the fluid was replaced with fresh serum from infected hosts (group 3), the cysts showed signs of regression by day 30 and became opaque, compared with the healthy transparent cysts in the controls (groups 1 and 2). From day 60-150, the laminated membrane of the treated cysts thickened, fluid was lost and the germinal membrane collapsed; the protoscolices within these cysts were degenerated and dead and failed to produce an infection when inoculated into susceptible animals.

Various hypotheses have been proposed to account for the survival of parasites in immunologically competent hosts⁶⁻¹¹. The data presented in this report confirm our working hypothesis and also offer a practical approach to the biological/medical treatment of *E. granulosus* cysts. Thus, in spite of the claim that hydatid cysts induce an insufficient adverse host response¹², our results indicate that circulating anti-*Echinococcus* antibodies can kill these cysts if they contact the inner surface of the germinal membrane in sufficient quantities. These results raise the possibility that the cyst membrane is either impermeable to cytotoxic antibodies and thus selectively permeable to macromolecules, or that these antibodies pass into the cysts in quantities too small to destroy the parasite. Since macromolecules of host origin have been demonstrated in hydatid cysts^{4,13,14}, we conclude that the latter mechanism is operative in hydatid infections and would explain the long persistence of hydatid cysts in the immunologically hostile environment of the host. Furthermore, a breakdown in the mechanisms controlling permeability of the membrane and

Table 1 Effect of the replacement of hydatid fluid by serum on the growth of *Echinococcus granulosus* cysts in cotton rats

Group	No. of animals	Treatment	No. of viable cysts Total no. of cysts
1	6	None	6/6
2	6	Normal cotton rat serum	6/6
3	6	Infected cotton rat serum	0/6

Adult cotton rats were each surgically transplanted with one cyst (1-3 cm in diameter) of *E. granulosus*. Two months later laparotomy was performed and the following treatments carried out: Group 1: no treatment; groups 2 and 3: the cysts were punctured with a 22 gauge needle and the fluid was aspirated and replaced by an equal volume of 20% ice-cold fresh serum (in Medium 199) from normal or heavily infected cotton rats (> 30 g cyst mass), respectively. The needle, an empty syringe and a syringe containing the diluted serum were fitted on to a 3-way valve (Pharmaseal) and used in the above procedure. Cyst growth and survival was determined every 30 d on laparotomy in alternate animals. The experiment was terminated on day 150 after treatment when the viability of the cysts was determined both macroscopically at necropsy as well as by an increase in cyst size and/or by the eventual formation of protoscolices in the sterile cysts after subsequent transplantation into susceptible animals.

the consequent leakage of cytotoxic antibodies into the cysts could account for dead hydatid cysts found sometimes in infected animals.

Although surgery is the commonest treatment for hydatid disease^{15,16}, it is often unsuccessful since spillage of cystic contents, particularly protoscolices, during surgical resection can result in anaphylactic shock and secondary hydatidosis. Furthermore, surgery is impractical when the cysts are embedded in vital organs, such as heart and brain, or when resection of the parasite also necessitates the excision of tissues that are vital to the host. Our findings suggest the possibility that a hydatid infection with *E. granulosus* can be treated and the parasite killed by replacing the fluid within the cysts with fresh serum from the infected host. Since the need for surgical resection would no longer be necessary, spillage of protoscolices and hydatid fluid is minimised and the host need not be subjected to ethanol, formalin or hypertonic saline which are currently used to kill the parasite before resection. Protoscolices that might escape as a result of this treatment would have been lysed by the complement proteins in the injected fresh serum. This new approach provides, for the first time, a practical immunotherapeutic method to treat and kill surgically non-resectable *E. granulosus* cysts.

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Identification of breast cancer transcortin and its inhibitory role in cell-mediated immunity

THE successful development and maintenance of a neoplastic malignancy is generally accepted to result from a temporary or lasting suppression of cell-mediated immune mechanisms. Many studies illustrate that whereas lymphocytes of a cancer patient are functionally sound, their participation in cell-mediated immune responses to an antigenic neoplasm is abrogated by factors present in the plasma¹. Among such factors identified are certain plasma proteins whose concentrations are markedly higher in the plasma of the cancer patient². We have shown that the content of transcortin, the cortisol-binding protein of human plasma, is higher in patients with demonstrable cancer³, and since transcortin inhibits the response of human lymphocytes to phytohaemagglutinin⁴, a test generally accepted as demonstrative of active cell-mediated potential, we have attempted to study the relationship of such elevated levels of plasma transcortin to breast cancer.

The question of whether serum obtained from freshly diagnosed breast cancer cases inhibits the response of normal human lymphocytes to the presence of phytohaemagglutinin is answered by the data presented in Table 1. The inhibition of the incorporation of ³H-thymidine into DNA promoted by such sera remains even after exhaustive dialysis, and therefore the responsible factor must be a species whose molecular weight is in excess of 10,000. In addition, we have tested the suppressive property of these sera on the incorporation of DNA precursors by lymphocytes of the RPM I-1788 cell line, and a similar inhibition was also found with all such sera tested. We also note that transcortin inhibits the incorporation of thymidine by either normal or RPM I-1788 lymphocytes.

Since breast cancer sera have been shown previously to contain two to three times as much transcortin as those of the corresponding normal females (our unpublished work), and since transcortin also inhibits the synthesis of DNA by human lymphocytes⁴, we investigated the relationship of increased levels of transcortin to the existence of the breast tumour; of primary concern was the question of the source of the increase in plasma transcortin. Previously, we used a transcortin-specific antibody tagged with fluorescence, which we applied to frozen sections of tissues.

Table 1 Effect of breast cancer sera and transcortin on the incorporation of ³H-thymidine by normal lymphocytes stimulated with PHA, and by RPM I-1788 lymphocytes

	N	Mean c.p.m. ± s.d.	% Difference
(1)* Normal lymphocytes stimulated with PHA			
Normal sera	5	6,680 ± 180	—
Breast cancer sera	10	4,290 ± 165	–36%
Transcortin	5	3,460 ± 51	–48%
(2)† RPM I-1788 lymphocytes			
Normal sera	5	2,065 ± 82	—
Breast cancer sera	10	842 ± 38	–60%
Transcortin	5	1,386 ± 18	–33%

*5 × 10⁵ Normal human lymphocytes were incubated in 2 ml of medium containing PHA with 0.2 ml of sera from normal or breast cancer patients, or with 50 mg of purified human transcortin⁴. At the end of 72 h incubation, 2 μCi of ³H-thymidine was added to each culture and incubation was continued for an additional 2 h.

†5 × 10⁵ RPM I-1788 lymphocytes were incubated in 2 ml of media with normal or breast cancer sera, or transcortin as above.

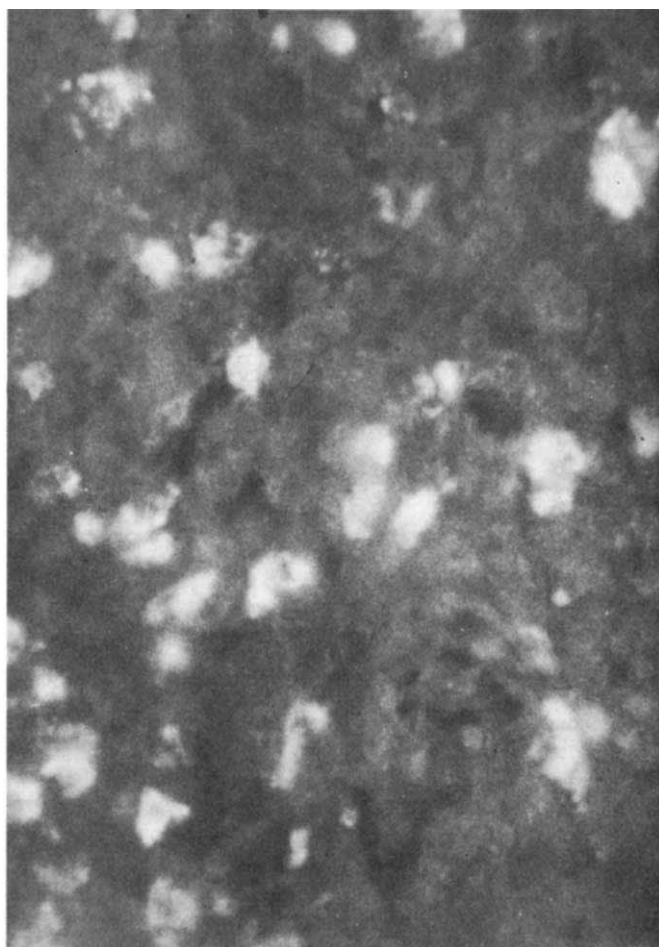


Fig. 1 Fresh biopsy breast tissues frozen rapidly and sectioned at a thickness of 4 μm. The sections were air dried and fixed in ice-cold acetone, and stored at –20 °C. Fluorescein-labelled transcortin antibody was applied as previously described⁴.

This permitted the *in situ* identification of intracellular transcortin in human liver⁵ and lymphocytes⁶ as well as in the syncytiotrophoblastic cells of the human placenta⁴. In the case of both the liver and the placenta, this molecule seems to be synthesised within the tissues studied and subsequently released to the immediate circulatory supply of the representative tissues. On the basis of the placental transcortin study, we reasoned that breast tissue might also demonstrate the *in situ* presence of a species recognisable by the fluorescein-labelled transcortin-specific antibody.

Figure 1 illustrates that transcortin is present within the sectioned cells of the human breast cancer tissue. No transcortin has been detected by this technique in normal breast tissue. We have also studied other breast diseases in this way and have noted that transcortin almost exclusively occurs in malignancies of the breast. Table 2 shows that whereas 52 normal breast tissues gave negative results, almost 95% of all breast cancers examined contained transcortin. It is interesting to note that the condition of gynaecomastia in the male, a benign tumour influenced by

Table 2 Fluorescent localisation of transcortin in breast lesions

	No. of cases	% Positive
Normal breast	52	0
Duct cell carcinoma	18	94
Lobular carcinoma	1	0
Fibroadenoma	12	8
Fibrocystic disease	16	13
Gynaecomastia	1	100

excessive oestrogen levels, is also positive for transcortin.

In an effort to define the functional role of breast cancer transcortin, we have tested the possibility that cytosols of breast cancer tissues could, as transcortin and breast cancer sera, limit the response of lymphocytes to PHA stimulation. Table 3 illustrates that only breast cancer cytosol exhibits appreciable inhibitory qualities in the incorporation of thymidine into the DNA of either normal or RPM I-1788 lymphocytes. The inhibitory factor is non-dialysable, and furthermore the inhibition can be obviated if cytosols are stripped of transcortin by previous incubation with the transcortin-specific antibody.

In summary, our studies indicate quite strongly that breast cancer tissue contains a transcortin-like species capable of limiting the response of human lymphocytes to PHA. Since oestrogens are known to induce breast cancer in both females⁷ and males⁸, and since oestrogen also

Table 3 Effect of normal and breast tumour cytosol on the incorporation of ³H-thymidine by PHA-stimulated human lymphocytes and RPM I-1788 lymphocytes

	No. of separate experiments	Average difference from controls (%)
(1) Normal lymphocytes stimulated with PHA		
Normal breast cytosol	11	-11 (±6)
Dialysed	1	-2
Tumour breast cytosol	11	-62 (±12)
Dialysed	2	-58
Transcortin	6	-50 (±2)
Dialysed	2	-48
(2) RPM I-1788 lymphocytes		
Control cultures	8	—
Cancer breast cytosols	6	-80
(3) Preincubation of breast cytosols with transcortin-specific antibody		
Normal breast cytosol		-10
Tumour breast cytosol		-8
Transcortin		-11

Breast tissues were obtained surgically, homogenised in 0.010 M Tris-phosphate pH 7.4, the lipid layer removed and cytosols prepared by centrifugation for 3 h at 105,000g. Protein concentrations were adjusted to 10 mg ml⁻¹. All cytosols prepared were used on that same day for all the above experiments. 0.2 ml of breast cytosols were added to cultures containing normal lymphocytes and PHA, or RPM I-1788 lymphocytes. Incubations were for 72 h at 37 °C. At the end of this period, 2 µCi of ³H-thymidine was added. Breast cytosols for some experiments were dialysed against 3 l of buffer for 24 h at 4 °C. In other experiments, breast cytosols were incubated with transcortin-specific antibody for 24 h at 4 °C. The antibody-antigen complexes were removed by centrifugation.

induces the biosynthesis of transcortin in liver⁹, the observation that the oestrogen-induced gynecomastic lesion also contains transcortin lends support to the hypothesis that oestrogen promotes the successful development and maintenance of a breast cancer by inducing the synthesis of transcortin within the abnormal cells. It is the release of this transcortin and its subsequent role in inhibiting the responses of T cells to the antigenic neoplasm which we believe to result in the successful maintenance of the breast cancer.

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Cyclic nucleotide-dependent phosphorylation of intestinal epithelium proteins

THE function of cyclic AMP as an important regulator of ion and fluid transport in the small intestine has been well documented¹. The non-dividing maturing crypt cells and the differentiated villous cells are the main sources of cyclic AMP-mediated secretion of water and electrolytes during experimental cholera in rats and guinea pigs^{2,3}. It has been proposed that cyclic AMP, analogous to its possible mode of action in kidney⁴ and toad bladder⁵ epithelial cells, controls ion movements across the apical membrane of the intestinal epithelial cell (enterocyte) by changing the state of phosphorylation of some protein(s) in the intestinal brush border⁶. Studies have shown, however, that adenylate cyclase^{7,8}, the high affinity form of particulate cyclic AMP phosphodiesterase (unpublished observation) and receptor-bound cyclic AMP (ref. 8) are localised predominantly in the basal-lateral membranes of the villous cell. In contrast, the luminal membrane is enriched in components of the cyclic GMP system, such as guanylate cyclase^{9,10}, cyclic GMP phosphodiesterase (unpublished observation) and receptor-bound cyclic GMP (ref. 8). The polarisation of both systems in the enterocyte would be more consistent with a model in which cyclic GMP, rather than cyclic AMP, is involved in the regulation of ion transport across the brush border, whereas cyclic AMP action might be confined to the contraluminal membrane. To test this hypothesis further, we have studied the subcellular distribution of specific substrate proteins for cyclic nucleotide-dependent protein kinases in isolated villous cells from rat small intestine.

Brush borders, microsomal fractions (enriched in basal-lateral membrane markers, compare ref. 11) and a 100,000g supernatant fraction (containing the cytosolic proteins) from villous cells were prepared as described previously^{2,9,12}. Subcellular fractions were incubated with ³²P-ATP in the presence of Mg²⁺, the proteins were separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and stained with Coomassie blue. Subsequently, the gels were dried and processed for autoradiography.

Protein staining patterns of brush-border preparations (Fig. 1) were characterised by a high content of an actin-like protein (molecular weight ~ 46,000) identified earlier by Tilney and Mooseker in microvillous cores from chicken intestine¹⁰. Incubation in the absence of cyclic nucleotides revealed a number of ³²P-labelled proteins, most of them not coinciding with major protein staining bands. Low concentrations of cyclic GMP (0.05-1.0 µM) or high concentrations of cyclic AMP (1.0-10 µM) caused an additional phosphorylation of a single brush-border protein (hereafter called protein B) with an apparent molecular weight of 86,000. The radioactivity of this band most probably reflects labelled phosphate esterified to serine or threonine residues of the protein because: no radioactive band could be detected using α-³²P-ATP; lipid extraction, treatment with hydroxylamine, cold or hot trichloroacetic acid or cold sodium hydroxide before electrophoresis did not influence the extent of labelling; and treatment with 0.4 M NaOH at 37 °C for 15 h removed the label almost completely (results

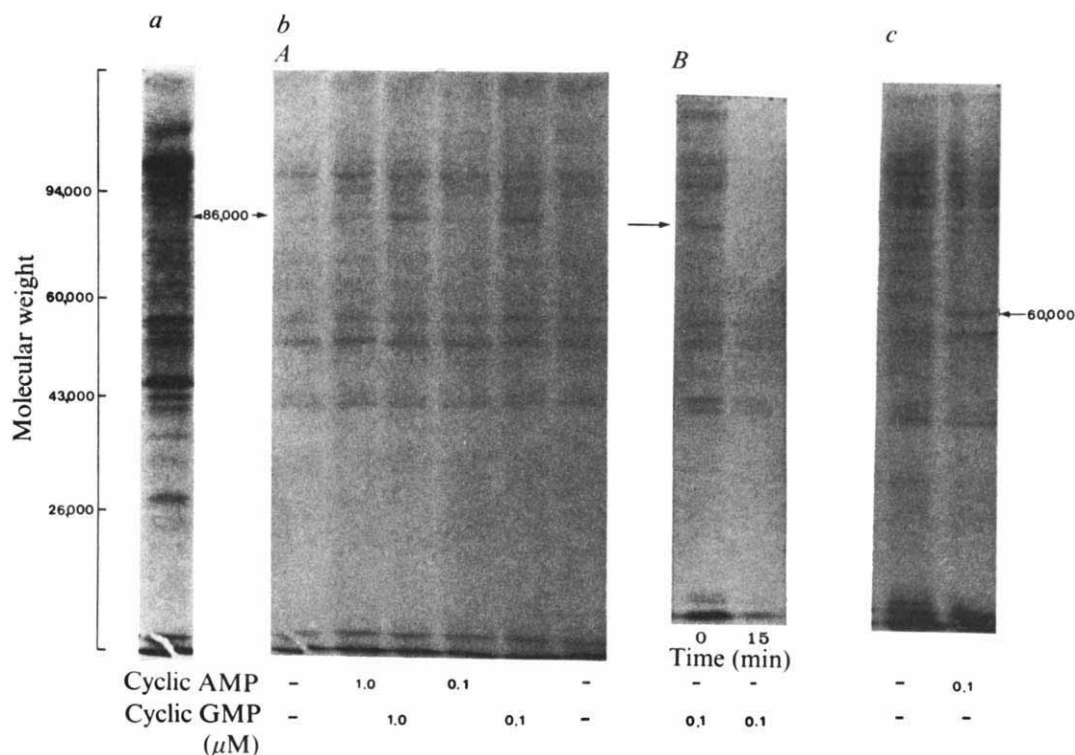


Fig. 1 *a*, Protein staining. *b* and *c*, Autoradiographs: Effects of cyclic GMP and cyclic AMP on endogenous phosphorylation of brush borders isolated from rat enterocytes. Intestinal brush borders (100–150 μ g of protein) without (*b*) or with (*c*) cytosolic proteins (60 μ g) were preincubated for 3 min at 30 °C in a volume of 50 μ l containing 40 mM histidine buffer, 50 mM manitol, 10 mM Tris-HCl, 5 mM sodium- β -glycerophosphate (to diminish degradation of ATP by alkaline phosphatase), 5 mM theophylline, 10 mM MgCl₂ and cyclic nucleotide concentrations as indicated (final pH 6.8). Phosphorylation (*A* and *c*) started by addition of 2 μ Ci γ -³²P-ATP (final concentration 20 μ M) and terminated after 10 s with 25 μ l of a solution containing 60 mM Tris-HCl, 2 mM ATP, 3% SDS and 30% glycerol (pH 7.4). To stop the reaction the mixture was heated immediately in a boiling water bath, cooled and provided with 0.5% β -mercaptoethanol. 40 μ l aliquots were loaded on 10% acrylamide–0.1% SDS slab gels (thickness 1 mm) with a 3% acrylamide stacking gel and run in the discontinuous buffer system of Laemmli¹³ using a Slabophor apparatus (Pleuger, Wijnegem, Belgium). Electrophoresis was carried out for 6–8 h at 30 mA. For the procedures of gel staining, drying and autoradiography we refer to Ueda *et al.*¹⁴. In the dephosphorylation experiments (*B*) brush borders were labelled first for 2 min at 0 °C as described above, followed by rapid sedimentation in an Eppendorf 3200 centrifuge (30 s, 10,000 r.p.m.). The pellet was suspended in 50 μ l of fresh medium prewarmed at 30 °C containing 10 mM MgCl₂, 5 mM ATP and 50 mM Tris-HCl (pH 7.0). After 5–15 min of incubation at 30 °C, dephosphorylation was terminated as described above. Molecular weight determinations were carried out according to Weber and Osborn¹⁵ using phosphorylase *a*, catalase, ovalbumin and chymotrypsinogen as markers.

not shown). The phosphoprotein B caused one of the major peaks in optical density tracings of brush-border autoradiographs but corresponded with only a minor band in the protein staining pattern (Fig. 1). Densitometric analysis also showed that incorporation of label into protein B proceeded linearly with time for 10 s at 30 °C or 30 s at 0 °C. Incorporation was much lower in the presence of 10 mM Mn²⁺ or Ca²⁺ and was nearly absent in the presence of Zn²⁺ (5 mM) or γ -³²P-GTP instead of ATP (data not shown). The apparent *K_a* value for its phosphorylation by cyclic GMP (0.06 μ M) was about 30-fold less than for cyclic AMP (2 μ M; Fig. 2). The endogenous dephosphorylation of protein B was a relatively slow process. At 30 °C, half of the ³²P label was removed after 5 min whereas 20% of the initial label was left after 15 min (Fig. 1). Dephosphorylation rates did not change by addition of cytosolic proteins or cyclic nucleotides. The different turnover rates of the phosphoproteins on the autoradiograph (Fig. 1) suggest that the intrinsic phosphatase(s) in the brush border possess a certain degree of specificity towards their substrates.

A cyclic GMP-dependent phosphorylation could not be detected in villous cell fractions other than brush borders. Presumably, protein B is an integral and specific component of the microvillous membrane. Its phosphorylation was still manifest in brush-border preparations purified according to Hopfer *et al.*¹⁷ with a relatively low content of core material (result not shown).

In spite of a wide variation in experimental conditions

we were unable to show any specific effect of cyclic AMP on protein phosphorylation in isolated brush borders. If a cytosolic fraction was included in the system as a possible source of cyclic AMP-dependent protein kinase, a specific cyclic AMP-dependent phosphorylation of a 60,000 molecular weight protein was decided (Fig. 1c), but a similar radioactive peak of equal intensity appeared if the same amount of cytosolic protein was incubated alone (Fig. 3c). The corresponding apparent *K_a* for cyclic AMP was 0.04 μ M (Fig. 2). A protein with similar properties was also found in microsomal membranes (Fig. 3b). This protein is probably identical to the so-called 'steroid and cyclic AMP regulated phosphoprotein'. (SCARP) detected by Greengard's group in cytosolic and membranous fractions from a large number of other rat tissues^{18,19}: its molecular weight is comparable to that of SCARP (49,000–54,000); it is seen as the highest labelled band on autoradiographs of cytosolic fractions (Fig. 3); replacement of Mg²⁺ by Zn²⁺ 5 mM or Ca²⁺ 2 mM led to inhibition instead of stimulation of the phosphorylation rate by cyclic AMP, both in cytosolic and microsomal preparation (data not shown); and cyclic GMP up to 10 μ M was entirely ineffective and was also unable to modify the cyclic AMP response (Figs 2 and 3). The only unusual property of the microsomal substrate protein was the strong stimulation of its phosphorylation by cyclic AMP in the presence of Mg²⁺. In particulate fractions from most other tissues (synaptic membranes excepted¹⁴), cyclic AMP was hardly effective in this condition or even displayed an inhibitory action (toad bladder⁸).

The present discovery of cyclic GMP-dependent phosphorylation of a specific microvillous protein provides a possible mechanism by which cyclic GMP might change the properties of transport systems exclusively at the luminal membrane of the villous cell. Phosphorylation of protein B might exert a direct effect on the ion transport properties of the membrane or, alternatively, might work rather indirectly by changing the contractility of the microvillous core. The latter mechanism could then be quite analogous to the action of cyclic GMP on smooth muscle contractility, most probably also mediated by changes in the phosphorylation state of specific membrane-bound substrate proteins for cyclic GMP-dependent protein kinases²⁰.

Since high concentrations of cyclic AMP are able to promote the phosphorylation of protein B as efficiently as cyclic GMP (Fig. 2) it may be proposed that brush border phosphorylation could also be changed by cyclic AMP in pathological conditions characterised by high levels of this nucleotide (cholera) or during *in vitro* incubations with high concentrations (10^{-3} – 10^{-2} M) of exogenously added cyclic AMP, dibutyl cyclic AMP or theophylline^{21,22}. Cyclic GMP levels might also be augmented in these conditions as a consequence of inhibitory or competitive effects

Fig. 2 Incorporation of 32 P label in endogenous substrate proteins as a function of cyclic nucleotide concentrations. *a*, Protein B (brush border); *b*, SCARP-like protein (cytosol). For experimental conditions, see Fig. 1. ■, Cyclic AMP; ▲, cyclic GMP. Absorbance of the 86,000 and 60,000 molecular weight bands on the autoradiographs were measured by scanning with a Vitatron Densitometer TLD 100 and peak areas were taken to be proportional to the amount of 32 P incorporated into each band.

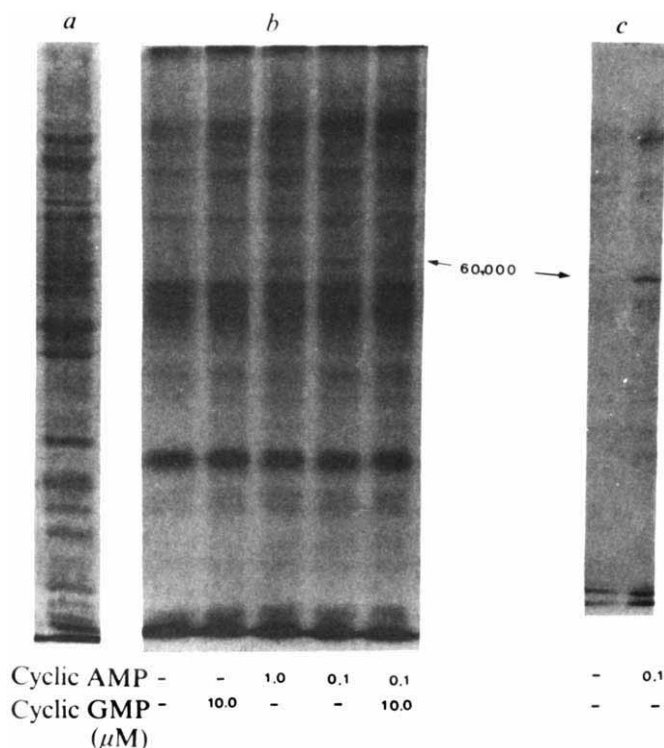
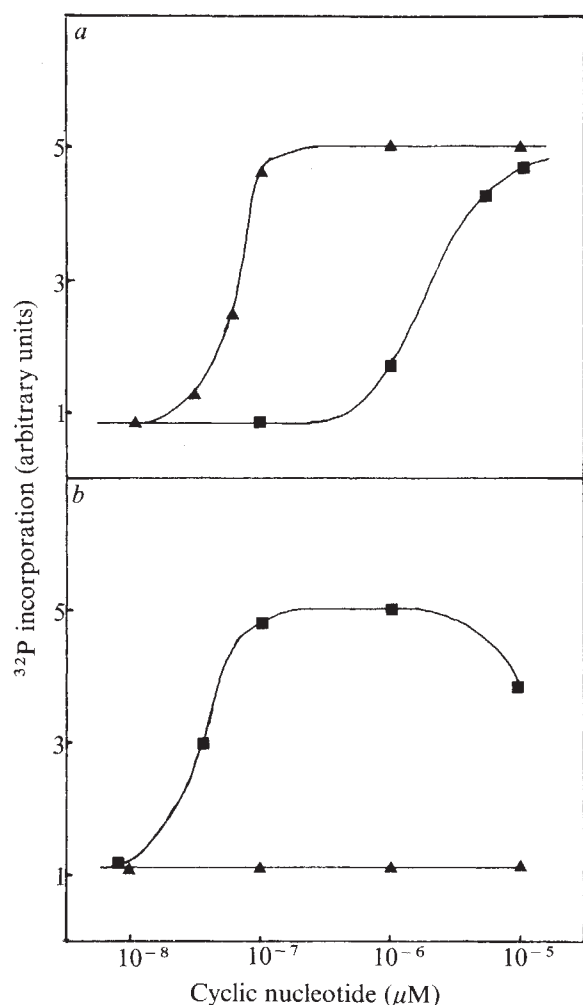


Fig. 3 Effects of cyclic AMP and cyclic GMP on the endogenous phosphorylation of microsomal (*a* and *b*) and cytosolic fractions (*c*) from isolated rat intestinal villous cells. Microsomes (200–250 μ g protein) or soluble fractions (containing 60–100 μ g protein) were phosphorylated for 15 s at 30 °C as described in Fig. 1. *a*, Protein staining; *b* and *c*, autoradiographs.

on cyclic GMP phosphodiesterases (unpublished and refs 23–25). In contrast, phosphorylation of the SCARP-like protein—most probably absent in the brush border but enriched in microsomal preparations—could then be involved in cyclic AMP-dependent changes of ion transport across the basal-lateral plasma membrane. Direct measurements of the degree of phosphorylation of protein B and the SCARP-like protein in intact epithelium in different hormonal conditions and absorption or secretion states will be necessary to further substantiate this model.

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In vitro inhibition of cyclic AMP phosphodiesterase by cortisol

HORMONAL effects on cyclic nucleotide phosphodiesterase activity are assumed to play an important role in the regulation of intracellular cyclic nucleotide concentrations¹. The respective hormones vary widely with respect to function and structure, and comprise polypeptides, biogenic amines, and steroids including the glucocorticoids^{2–4}. In no case, however, has the mechanism of hormonal action on cyclic AMP phosphodiesterase clearly been established. It is not known whether the enzyme itself is the primary point of attack by the hormone or whether the observed changes in enzyme activity (for review, see ref. 1) are side-effects of hormonal action, since no conclusive *in vitro* studies have been undertaken so far.

In the course of experiments designed to elucidate the possible function of the adrenal glucocorticoids as stimulators of the cyclic AMP-mediated human chorionic gonadotropin (hCG) sensitivity of the postnatal Leydig cell in the rat⁵, we have tested the influence of cortisol on cyclic AMP phosphodiesterase activity in crude testis homogenates. For comparison, we have

Fig. 1 Inhibition of cyclic AMP phosphodiesterase from rat testicular tissue. Dixon plot of the reciprocal of reaction velocity against cortisol concentration. Each point represents the mean of duplicate determinations. Cyclic AMP concentrations in the assay were 0.120 (●), 0.058 (■), and 0.036 (▲) mM. Pooled testes from 20-d-old SIV 50 rats were homogenised with a motor-driven glass pestle in 10.9% sucrose 1:10 (w/v). The homogenate was sonicated with a Branson B12 sonifier at setting 1 for 30 s. All steps were carried out in an ice bath. The homogenate was adjusted to pH 6.0 with 1 M acetic acid⁶, and a clear supernatant was obtained by centrifugation at 20,000g at 4 °C for 20 min.

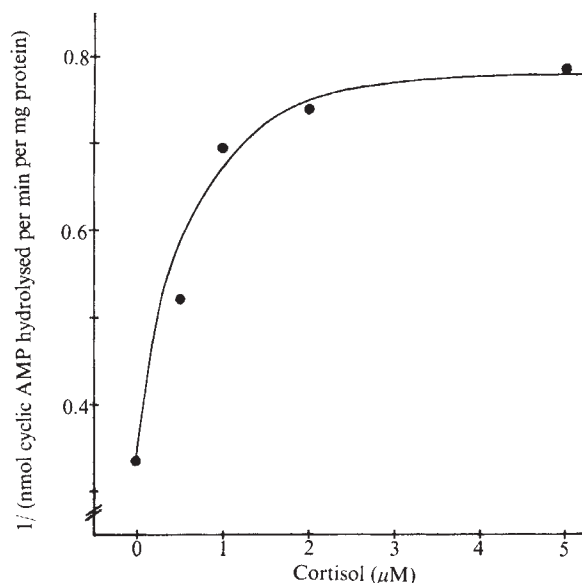
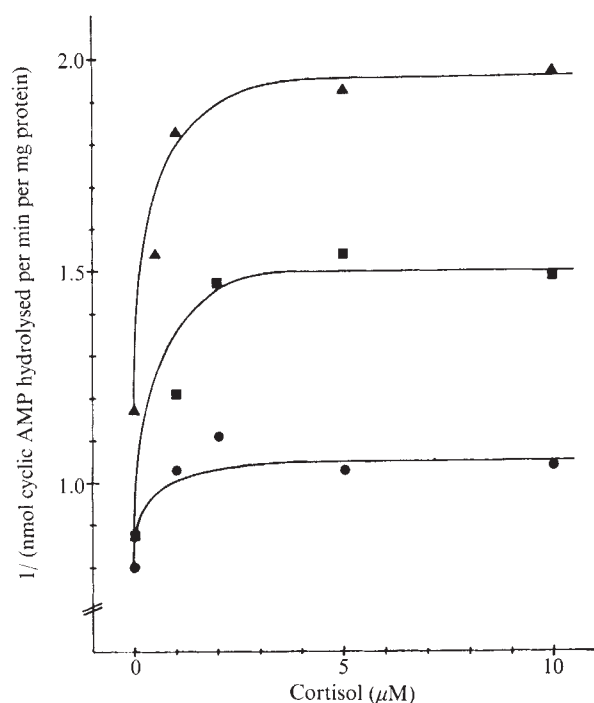


Fig. 2 Inhibition of cyclic AMP phosphodiesterase from beef heart. Dixon plot of the reciprocal of reaction velocity against cortisol concentration. Each point represents the mean of duplicate determinations. Cyclic AMP concentration in the assay was 0.0124 mM. The substrate was solved in 66 mM Tris-HCl buffer, pH 8.5, containing 1 mM MgCl₂.

examined the effect of cortisol on the activity of the commercially available enzyme purified from beef heart.

Cyclic AMP phosphodiesterase was assayed as described by Rutten *et al.*⁷. The incubation medium contained 66 mM Tris-HCl, pH 8.5, 1 mM MgCl₂, 0.5 μM ³H cyclic AMP (Amersham Buchler, specific activity 27 Ci mmol⁻¹), varying concentrations of unlabelled cyclic AMP (Boehringer, Mannheim, Germany), and 25 μl diluted tissue homogenate supernatant or enzyme solution in a final volume of 140 μl. Homogenate supernatant and enzyme concentrations were adjusted to a total substrate conversion not exceeding 10% of the initial concentrations. After 20 min incubation at 37 °C (the reaction was at least 2 h linear) the reaction was stopped by placing the tubes into boiling water for 2 min; 8 mU 5' nucleotidase (Sigma) were added, the mixture was centrifuged (10 min at 10,000 g) and 100 μl of the supernatant were applied to a column (20 × 6 mm) filled with DOWEX 200-400 mesh (Serva). The column was eluted with 10 ml 0.1 M NaHCO₃, the eluate mixed with 10 ml scintillation fluor (Sztintgel; Roth, Karlsruhe, Germany) and counted in a Tri-Carb scintillation counter. Inhibition experiments were carried out with homogenate supernatants as well as with enzyme solutions preincubated with 0.5–10 μM cortisol (Merck, Darmstadt, Germany) at 37 °C for 20 min. The ethanol, in which cortisol had been dissolved, was evaporated before addition of the enzyme solutions, since the presence of ethanol heavily interfered with the phosphodiesterase assay. The phosphodiesterase assay mixture was adjusted to the corresponding concentrations of cortisol. Controls, containing no cortisol, were preincubated and assayed in parallel.

The results of our experiments demonstrate that both testicular and beef heart cyclic AMP phosphodiesterase can be inhibited by cortisol at low concentrations. In Figs 1 and 2 the reciprocals of the reaction velocities are plotted against cortisol concentration⁸. The curves obtained can be easily interpreted as a partially competitive type of inhibition. This type of inhibition occurs when an intermediate complex of enzyme–substrate–inhibitor is formed, which then breaks down at the same rate as the enzyme–substrate complex, the inhibition therefore being purely an effect on affinity (Fig. 3). Assuming that product formation is rate-limiting and the enzyme, enzyme–substrate, enzyme–inhibitor, enzyme–inhibitor–substrate are rapidly equi-

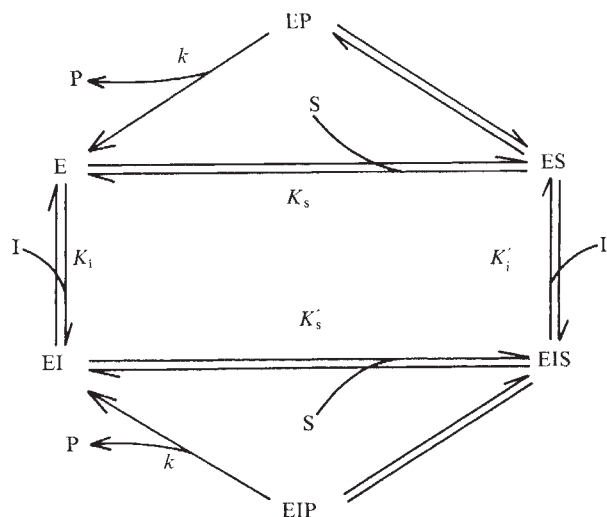


Fig. 3 Reaction scheme: partially competitive inhibition. E, Enzyme; EP, enzyme-product; EI, enzyme-inhibitor; EIP, enzyme-inhibitor-product; P, product; S, substrate; ES, enzyme-substrate; I, inhibitor.

brating, the 'rapid equilibrium treatment' easily leads to the rate equation⁹

$$v = \frac{V}{1 + \frac{K_m(1 + i/K_i)}{s(1 + (i/K_i)(K_m/K'_s))}}$$

The plateau of the resulting curve is attained when all the enzyme is combined with inhibitor, and the degree of inhibition can increase no further.

Electrophoretic investigations of testis homogenates from 20-d-old rats revealed a single enzyme species possessing cyclic AMP phosphodiesterase activity. This enzyme exhibited true Michaelis-Menten behaviour in the range of substrate concentrations tested (0.6 mM–4.5 μ M). Before the inhibition constants could be calculated, K_m and V had to be determined. We found the specific activity to be 1.5 mU per mg protein and the Michaelis constant to be 0.018 mM. Our results are largely consistent with the data reported by other workers¹⁰. The K_m and the specific activity of the commercially available phosphodiesterase purified from beef heart were 0.13 mM and 37 mU per mg protein, respectively. The range of substrate concentrations tested was 0.36 mM–2.9 μ M.

K_i and K'_s were calculated by fitting a curve to the experimentally determined values of Figs 1 and 2 in an iterative procedure making use of an appropriate computer program. Subsequently the equilibrium constant K'_i was determined according to the equation

$$K_i/K'_i = K_s/K'_s$$

Values of K_m , K'_s , K_i and K'_i for the enzymes from rat testis and beef heart are summarised in Table 1.

Thus, high K_m cyclic AMP phosphodiesterases from two different sources, regardless of purity, seem to be inhibited by the same mechanism at low concentrations of cortisol. We have

recently shown that low K_m cyclic AMP phosphodiesterase present in the testicular tissue of the 10-d-old rat can be inhibited by cortisol in a manner indicative of the partially competitive type of inhibition (W. E., M. F., J. Frowein and J. S., unpublished). It is tempting, therefore, to speculate that many of the so-called 'permissive effects' produced by glucocorticoids on cyclic AMP-mediated processes^{11,12} reflect in fact a direct interaction of these hormones with cyclic AMP phosphodiesterase³.

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(–)-Propranolol inhibits the behavioural responses of rats to increased 5-hydroxytryptamine in the central nervous system

THERE are now several reports of the beneficial effect of large doses of ($\pm$)-propranolol in the treatment of various psychoses, particularly schizophrenia^{1–3}. These reports prompted us to investigate whether propranolol had any effect on the behavioural syndrome produced in rats by increasing the functional activity of 5-hydroxytryptamine (5-HT) or dopamine (DA) in the central nervous system. Our results indicate that large doses of (–)-propranolol but not (+)-propranolol inhibit the behavioural responses resulting from increased functional activity of central 5-HT but not DA.

To investigate the action of propranolol on brain 5-HT function we used a behavioural model⁴ reviewed elsewhere⁵. This involves the injection of tranlycypromine and L-tryptophan and measurement of the hyperactivity that results from the increased synthesis of 5-HT and its "spill-over" into functional activity.

Rats were given ($\pm$)-propranolol (40 mg kg⁻¹) by intraperitoneal injection or saline and 45 min later given tranlycypromine followed by L-tryptophan (details in legend to Fig. 1). All aspects of the hyperactivity syndrome were almost totally inhibited by ($\pm$)-propranolol pretreatment (Table 1).

No evidence was found for alteration of the steady-state concentration of whole brain 5-HT, 45 min after ($\pm$)-propranolol (40 mg kg⁻¹). Both the rate of 5-HT synthesis and the rate of accumulation of 5-HT following administration of tranlycypromine and L-tryptophan were also unaltered by the single injection of ($\pm$)-propranolol. This demonstrated that propranolol was not exerting its effect on the hyperactivity syndrome by altering 5-HT biosynthetic mechanisms and suggested that the drug was in some way altering postsynaptic responses to 5-HT receptor stimulation.

Table 1 Equilibrium constants for enzymes from rat testis and beef heart

	K_m (mM)	K'_s (mM)	K_i (μ M)	K'_i (μ M)
Rat testis	0.018	0.075	0.05	0.21
Beef heart	0.13	0.37	0.15	0.43

This was examined indirectly by injection of the suggested 5-HT agonist 5-methoxy *N,N*-dimethyltryptamine (5-MeODMT). This compound produces a hyperactivity syndrome qualitatively identical to that seen when tranlycypromine and L-tryptophan are given but with a different time course and it has been suggested that this compound stimulates central 5-HT receptors⁸.

Pretreatment of rats with ($\pm$)-propranolol (40 mg kg⁻¹) 45 min before tranlycypromine (20 mg kg⁻¹), with 5-MeODMT (2 mg kg⁻¹) given 30 min later resulted in almost complete abolition of the hyperactivity response. A similar reduction in the hyperactivity was seen in rats pretreated with ($\pm$)-propranolol (40 mg kg⁻¹) following subsequent administration of tranlycypromine and 5-methoxytryptamine, another suggested 5-HT agonist^{7,8}. These results indicated therefore that propranolol inhibits the behavioural changes produced by central 5-HT stimulation and that it probably does so by means of a postsynaptic 5-HT receptor mechanism or through an effect on neuronal structures connected to these receptors.

The hyperactivity once established could be considerably reduced within 5 min by injection of ($\pm$)-propranolol (40 mg kg⁻¹) (Fig. 1). Furthermore, no inhibition was observed when ($\pm$)-propranolol (40 mg kg⁻¹) was given 3 h before the tranlycypromine administration. Since the half life of propranolol is about 40 min (ref. 9) these results suggested that the effect of propranolol was due to the parent compound rather than a metabolite. This view was strengthened by the observation that pretreatment of the rats with the drug metabolising enzyme inhibitor SKF525A, while not altering the hyperactivity seen when tranlycypromine and tryptophan were given, did make ($\pm$)-propranolol effective in inhibiting the hyperactivity at a dose (20 mg kg⁻¹) which is normally almost ineffective (Table 1).

The effect of the two isomers was next examined to determine whether both forms of the drug were equally active in inhibiting the 5-HT induced hyperactivity

Table 1 Effect of ($\pm$)-propranolol pretreatment on hyperactivity induced by tranlycypromine and L-tryptophan

Injected	Total movements during 90 min following L-tryptophan (50 mg kg ⁻¹)
Saline	5,316 $\pm$ 598 (8)
($\pm$)-Propranolol (40 mg kg ⁻¹)	886 $\pm$ 284 (3)*
(-)-Propranolol (20 mg kg ⁻¹)	1,041 $\pm$ 357 (3)*
(+)-Propranolol (20 mg kg ⁻¹)	4,558 $\pm$ 319 (3)
($\pm$)-Propranolol (20 mg kg ⁻¹)	4,090 $\pm$ 759 (4)
($\pm$)-Propranolol (20 mg kg ⁻¹) + SKF 525A (2 $\times$ 10 mg kg ⁻¹)	1,740 $\pm$ 180 (3)†
Pronethalol (40 mg kg ⁻¹)	3,631 $\pm$ 475 (4)†
Practolol (40 mg kg ⁻¹)	4,728 $\pm$ 920 (4)
Butoxamine (40 mg kg ⁻¹)	5,355 $\pm$ 1,031 (3)
	Total movements during 60 min following L-dopa (50 mg kg ⁻¹)
Saline	3,140 $\pm$ 180 (3)
(-)-Propranolol (20 mg kg ⁻¹)	3,305 $\pm$ 110 (3)

Rats were injected with propranolol, pronethalol, practolol or butoxamine 45 min before tranlycypromine and activity measured following subsequent L-tryptophan or L-dopa administration as described in caption to Fig. 1. All drugs were dissolved in 0.9% saline except practolol which was suspended in 0.9% saline containing 1% carboxymethylcellulose and all these drugs were injected intraperitoneally. In the SKF 525A experiment rats were injected subcutaneously with SKF 525A (10 mg kg⁻¹) 30 min before and 30 min after the propranolol administration, tranlycypromine being given 45 min after propranolol as before. Results expressed as mean $\pm$ s.e.m. with number of observations in brackets.

*Different from saline $P < 0.01$.

†Different from saline $P < 0.05$.

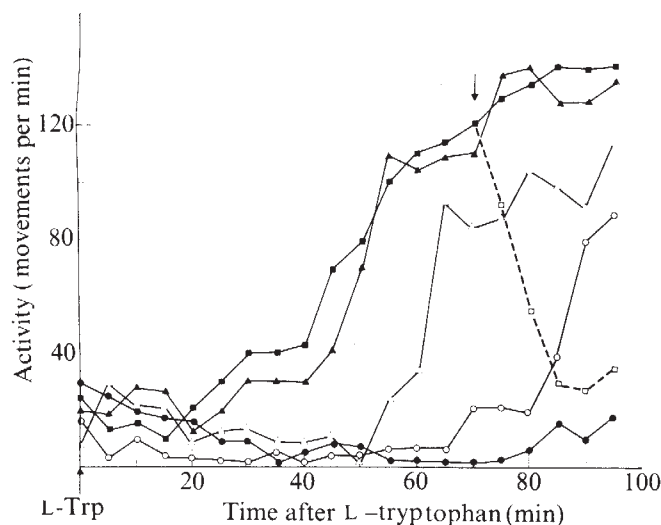
‡Different from ($\pm$)-propranolol (20 mg kg⁻¹) $P < 0.01$.

response. Injection of (-)-propranolol (20 mg kg⁻¹) 45 min before the tranlycypromine administration abolished the hyperactivity following subsequent tryptophan injection as effectively as when ($\pm$)-propranolol (40 mg kg⁻¹) had been given (Fig. 1 and Table 1) and the hyperactivity was inhibited by (-)-propranolol in a dose dependent manner (Fig. 1). Injection of (+)-propranolol (20 mg kg⁻¹) however was without effect on the hyperactivity (Fig. 1) suggesting that it is only the (-)-form of the drug in the racemate that is inhibiting the 5-HT induced behavioural changes.

To find out if propranolol had any effect on brain dopaminergic function we examined whether propranolol administration altered the locomotor activity produced by administration to rats of tranlycypromine and L-dopa since this activity seems to be almost solely the result of stimulation of dopaminergic systems in the brain¹⁰. Administration of ($\pm$)-propranolol (40 mg kg⁻¹) or (+) or (-)-propranolol (20 mg kg⁻¹) 45 min before the tranlycypromine did not alter the locomotor activity seen after subsequent L-dopa administration (Fig. 2). This indicated that propranolol has no inhibitory action on DA neurotransmission, a conclusion strengthened by two further observations. First (-)-propranolol (20 mg kg⁻¹) did not inhibit the rotation produced by amphetamine (5 mg kg⁻¹) in unilateral nigrostriatal lesioned rats and it is believed that turning produced in these animals by amphetamine is the result of dopaminergic stimulation^{11,12}. Furthermore, propranolol is ineffective in inhibiting striatal dopamine sensitive adenylate cyclase¹³, a finding confirmed in our current studies. It was also found that ($\pm$)-propranolol (40 mg kg⁻¹) had no effect on brain DA or noradrenaline concentration 45 min later.

The effect of other β -adrenergic receptor blocking drugs was also investigated. Propranolol is a β_1 and β_2 receptor blocking agent, and being lipid soluble enters the brain. The structurally related drug pronethalol (40 mg kg⁻¹) was almost ineffective in inhibiting the 5-HT mediated response, as was butoxamine (40 mg kg⁻¹) a lipid soluble β_2 antagonist (Table 1). Practolol (40 mg kg⁻¹) a β_2 receptor blocking agent that does not enter the brain in significant quantity¹⁴ did not inhibit hyperactivity (Table 1). Thus propranolol

Fig. 1 Effect of (+)- and (-)-propranolol on the hyperactivity produced by administration of tranlycypromine and L-tryptophan. Groups of 3 rats (male Sprague-Dawley, 160–200 g, Anglia Laboratory Animals, Huntingdon) were injected with either saline (■), (-)-propranolol, 20 mg kg⁻¹ (●), 15 mg kg⁻¹ (○), 10 mg kg⁻¹ (△) or (+)-propranolol, 20 mg kg⁻¹ (▲). After 45 min all groups were injected with tranlycypromine (20 mg kg⁻¹) and L-tryptophan (L-Trp; 50 mg kg⁻¹) 30 min later. Fig. 1 shows subsequent changes in activity in each group using LKB Animex activity meters (sensitivity and tuning: 30 μ A). The behavioural changes observed and method of measurement were exactly as described previously⁴. The effect of administration of (+)-propranolol (40 mg kg⁻¹) to rats already displaying hyperactivity is also shown (□), the drug being administered at the arrow.



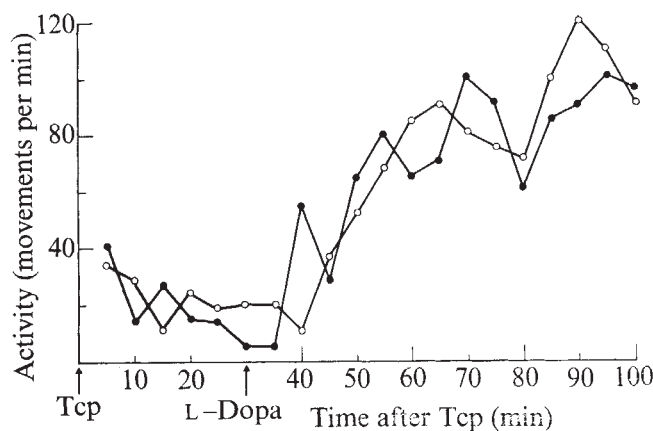


Fig. 2 Effect of (–)-propranolol on the locomotor activity produced by administration of tranylcypromine (Tcp) and L-dopa. Groups of 3 rats were injected with either saline (●) or (–)-propranolol, 20 mg kg⁻¹ (○). After 45 min both groups were injected with tranylcypromine (20 mg kg⁻¹) with L-dopa (50 mg kg⁻¹) 30 min later. The figure shows subsequent changes in activity. The behavioural changes observed were as described previously¹⁰.

was the only one of the β -adrenergic blocking drugs tested to inhibit 5-HT induced hyperactivity. Furthermore, this action cannot be ascribed to any tranquillising action of propranolol, since previous studies in rats have shown both isomers to be equipotent in this respect^{15,16}.

The results therefore all suggest that propranolol inhibits central 5-HT function, a finding that is analogous to the recent observation that (–)-propranolol (but not the (+)-isomer) blocks neurotransmission produced by 5-HT in the cat cervical ganglion¹⁷.

If our conclusions are correct that propranolol inhibits central 5-HT neurotransmission and if future studies confirm its efficacy in the treatment of schizophrenia then there are several important implications.

Much interest has been focused on the dopamine receptor blocking action of neuroleptics as their mode of action, as anti-psychotic drugs^{18,19} and indeed this has led to suggestions that increased activity of dopamine pathways may be involved in the aetiology of schizophrenia. If propranolol inhibits the central effects of 5-HT but not dopamine and if it is effective by this mechanism in schizophrenia then indirectly this points to an involvement of 5-HT as well as, or instead of, dopamine in this condition. Indeed, if 5-HT mechanisms were involved at a higher organisational level than dopaminergic mechanisms then the action of current neuroleptics could still be explained by their action on dopaminergic mechanisms. In rats inhibition of dopaminergic function either by use of neuroleptic drugs^{6,20} or the decrease of brain dopamine concentration²¹ blocks the behavioural responses to increasing 5-HT functional activity in the brain. Thus it seems that blocking dopaminergic activity can result in inhibition of certain aspects of central 5-HT function.

The demonstrated effect of propranolol on central 5-HT function, raises the question as to whether this is the mode of its action as an anti-schizophrenic drug and, if so, the role of 5-HT in schizophrenia.

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Decreased uptake of 5-hydroxytryptamine in blood platelets from depressed patients

THE search for biological aberrations in affective disorders has been intensified within the last few years¹. Special attention has been given to the changes in the metabolites of 5-hydroxytryptamine (5-HT) and of catecholamines in the cerebrospinal fluid². In this laboratory³ as well as in several others^{4,5} the blood platelet has been used as an easily obtainable model of 5-HT neurones. Studies on the storage, metabolism and uptake of 5-HT in various neurological and mental illnesses have been largely negative^{1,6,7} except in Down's syndrome^{8–10}. Neither in schizophrenia⁶ nor in affective illness⁷ have any changes been found. By using low substrate concentrations and measuring the initial uptake rate, both the uptake kinetics in platelets and the inhibitory potencies of antidepressant drugs were recently shown to correlate well with those found in synaptosomes¹¹. It was therefore of considerable interest to examine the kinetics of 5-HT uptake anew, in the platelets of certain psychiatric patients, notably those with manic-depressive illness. This report indicates that in the platelets of depressed patients the uptake of 5-HT is clearly decreased.

The patients studied were newly hospitalised with a diagnosis of depression. None of them had used tricyclic antidepressants or any other drugs known to interfere with the amine transport mechanisms for at least two days before the study commenced and some of them had never used antidepressants. Nor were any differences observed between patients who had recently ingested drugs and those who had not. The controls were healthy laboratory personnel, and the sample was taken at the same time as that of the patient. Platelets from one patient and one control were usually examined simultaneously.

The experiments were performed essentially as described previously¹¹. Blood platelets were isolated and incubated in Krebs–Henseleit bicarbonate buffer with tritiated 5-HT. The accumulation of radioactive 5-HT to platelets in 5 min was determined. After subtracting the portion taken up by non-active processes, the data were subjected to kinetic analysis¹².

A very clear and highly significant difference was found in the 5-HT uptake by platelets from patients as compared to those from the controls (Fig. 1). The initial uptake by the platelets of depressed patients was about half as active as that of the controls. This is also reflected in the V_{max} of the process (Fig. 2). On the other hand, no difference was noted in the K_m . This suggests that there are less of the hypothetical transport molecules or ‘‘amine carriers’’^{13,14} in the cell membranes, or that some of them are

Fig. 1 Uptake of 5-HT by blood platelets of patients with depression (■) and controls (●) when the 5-HT concentration was varied from 10^{-7} to 10^{-4} M. The continuous thick lines show the total accumulation which continued linear to 10^{-4} M (thin lines indicate extrapolation from the highest concentrations). The dotted lines show the active uptake after subtraction of the linear part¹¹. Before the incubation, the platelet-rich plasma was diluted with autologous platelet-free plasma to contain 200,000 platelets mm^{-3} . An aliquot (100 μl) of diluted plasma (containing 0.15% EDTA) was added to 3.9 ml of prewarmed (37 °C) Krebs-Henseleit bicarbonate buffer (pH 7.4), containing 10^{-8} M ^3H -5-HT (5.66 Ci mmol^{-1}) and a necessary amount of cold 5-HT to make up total 5-HT concentrations of 10^{-7} to 10^{-4} M. After 5 min of incubation in a metabolic shaker, the platelets were separated by filtration (cellulose nitrate filters, pore size 0.2 μm) and washed with 10 ml of ice-cold saline. The radioactivity accumulated to the filters was assayed by liquid scintillation. Means $\pm$ s.e.m. of duplicate experiments on 10 patients and 10 controls.

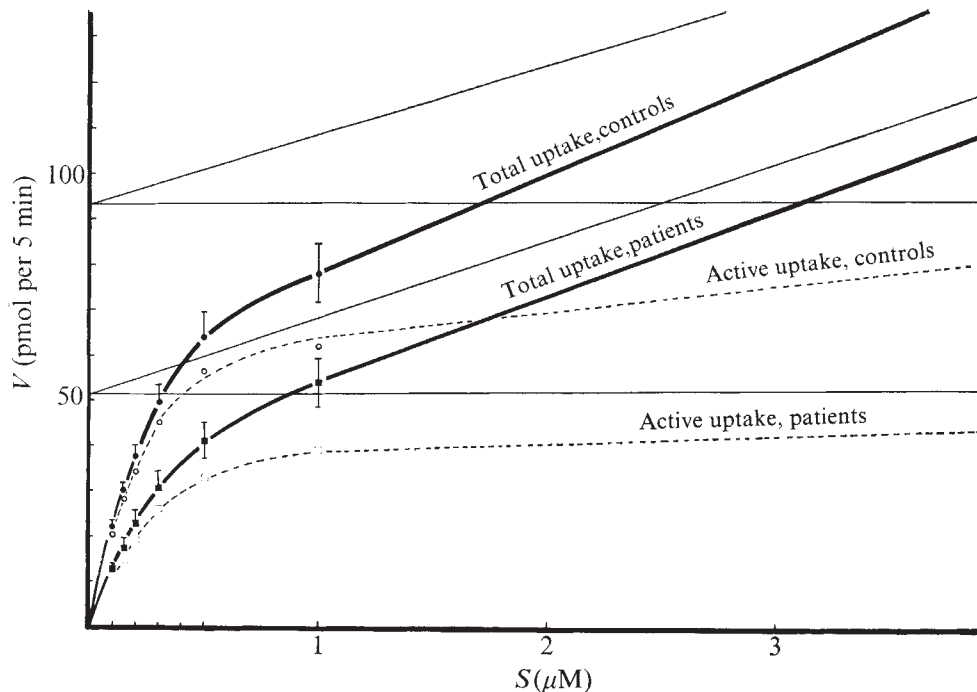
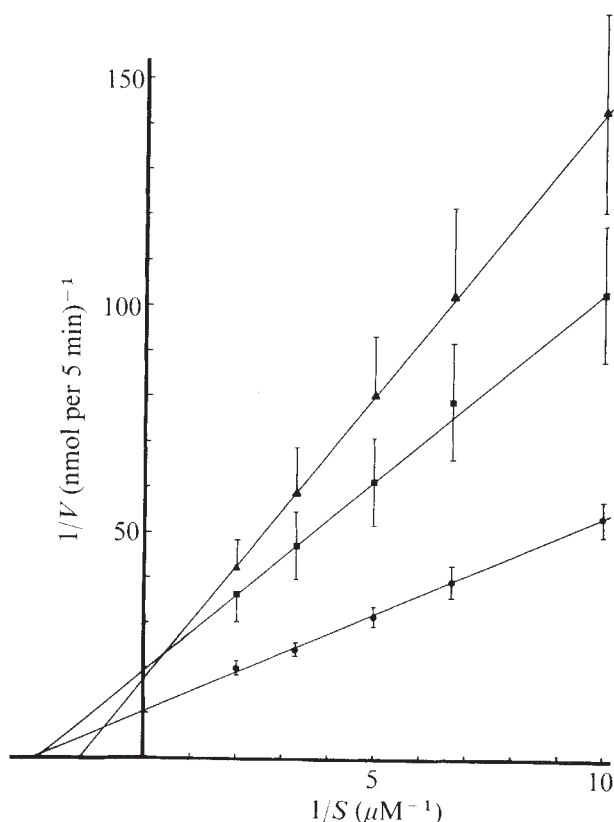


Fig. 2 Double-reciprocal plots of 5-HT uptake by platelets from controls (●), from untreated patients (■) and from patients treated for 4 weeks with imipramine or amoxepine (▲). The plots were calculated by the method of least squares. The kinetic data were: controls V_{max} 92 pmol per 2×10^7 platelets per 5 min, K_m 0.40 μM ; untreated patients V_{max} 49 pmol per 2×10^7 platelets per 5 min, K_m 0.41 μM ; treated patients V_{max} 59 pmol per 2×10^7 platelets per 5 min, K_m 0.75 μM . Conditions as in Fig. 1.



in an inactive state. Those which remain, however, are in a fully functional state as indicated by the unchanged K_m . The change is quite contrary to that seen in platelets or in brain synaptosomes after exposing them to tricyclic drugs¹¹: they increase the K_m but the V_{max} remains the same, that is, the affinity to the transport is decreased, but the number of hypothetical carrier molecules remains the same. Also in the present study the V_{max} remained the same, but the K_m increased, when imipramine 10^{-8} M was added to the incubation medium. The same change was caused by imipramine both in the patients and in the controls. The different mode of kinetics rules out the possibility that the decrease in 5-HT uptake would be due to some tricyclic drug in the plasma and in the platelets of these patients.

After 4 weeks' treatment with antidepressants (either imipramine or amoxepine) a change in uptake characteristics was observed. The V_{max} had moved towards the control. At the same time, however, an increase in the K_m , apparently due to the antidepressant, was noted (Fig. 2). Patients under chronic lithium treatment, but at present without depressive symptoms, did not differ from the controls (J.T., E.T., Ahlfors and Levä, to be published).

We feel that these findings are most exciting and might be investigated along two lines: first, to find out whether it is feasible to use these aberrations as a basis for laboratory tests for certain subtypes of depression, and second, what these changes imply about the functioning of tryptaminergic neurones in the brain. If a similar change could be demonstrated in the neurones, the most crucial question would be whether this decrease in the 5-HT uptake is a constitutional change typical of the individual and common to both platelets and neurones, which seem to have the same or very similar mechanism to take up 5-HT^{5,11}. On the other hand, the change could also be secondary and, possibly, compensatory to depression. In that case a question is how the change is transmitted to platelets. Perhaps hormonal or some other chemical regulatory mechanism might then be considered. Either way, these results may add a new dimension to the biological parameters in affective illness.

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Reduction of quantum contents of synaptic potentials after postsynaptic axotomy

THE loss of synaptic transmission in the chick ciliary ganglion that is observed after the postganglionic section of the ciliary nerves is accompanied by both a decrease in acetylcholine (ACh) sensitivity of the axotomised cells and a change in the transmitter release characteristics of the presynaptic terminals^{1,2}. Evidence for the presynaptic change included a decrease in the mean quantum content m of the excitatory postsynaptic potentials (e.p.s.p.s) and a greater susceptibility to fatigue during repetitive stimulation. The reduction in quantum content after axotomy was inferred from an increase in the coefficient of variation of the e.p.s.p. amplitude distribution in the depressed state. In the experiments reported here it was possible to measure miniature synaptic potentials (m.e.p.s.) in axotomised preparations so that more accurate estimates of the mean quantum content of e.p.s.p.s could be made.

Experiments were done on 3- and 4-d-old chickens. Axotomy of the ciliary nerves was performed within a few hours after hatching. Surgical and experimental procedures have been described before^{1,3}. To ensure that transmission failure in axotomised preparations was not caused by disuse of the ganglionic synapses, in some chickens only the eyelids were sutured together and the animals were kept and fed in the dark for 4–5 d. No impairment of transmission was observed in ganglia taken from these control animals.

The m of the e.p.s.p.s was estimated by dividing their mean amplitude ($\bar{v}$) by the mean quantal size ($\bar{v}_1$). Mean e.p.s.p. amplitudes were determined from 5–15 measurements, taken at 10-s intervals to avoid synaptic depression. In the axotomised cells action potential initiation by the e.p.s.p.s could be blocked by hyperpolarising the cell (Fig. 1a). In some normal cells with large e.p.s.p.s it was not possible to pass sufficient hyperpolarising current to achieve such block. In such cases the e.p.s.p. amplitude was estimated from the inflexion at the onset of the action potential (Fig. 1b, arrow) or, if no such inflexion was present, from the residual synaptic depolarisation after the action potential (Fig. 1c, arrow). The latter two measurements represent underestimates of the true e.p.s.p. amplitudes. Quantal sizes were determined in two ways. In most experiments spontaneous release of quanta could be induced by stimulating the preganglionic nerve at 30–50 Hz for 30 s. The modal amplitude of the spontaneous m.e.p.s.p.s was taken as the quantal

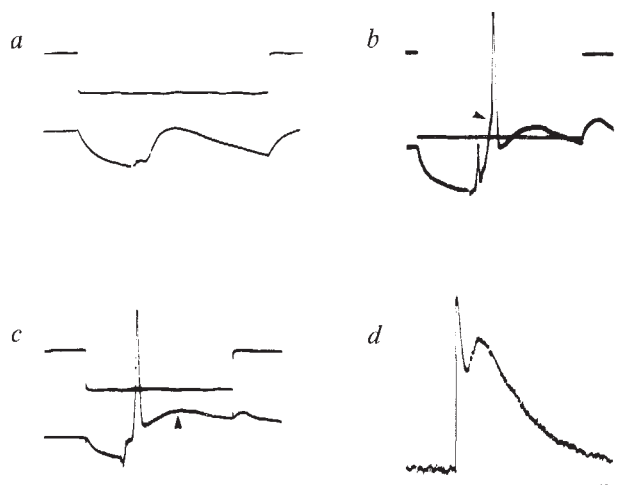


Fig. 1 Examples of e.p.s.p.s (used for calculation of mean quantum contents) from axotomised (*a* and *d*) and control cells (*b* and *c*). Amplitudes were corrected for nonlinear summation⁹, neglecting the influence of membrane capacitance. The resulting overestimation of the mean quantum contents, m , were, at least partially, balanced by the underestimated measurements of e.p.s.p. amplitudes, when the generation of action potentials could not be blocked. Occasionally, e.p.s.p.s in such cells were large enough to initiate two action potentials. Calibration: 40 mV (*a*, *b* and *c*), 4 mV (*d*); 10 ms.

size. In some of the axotomised cells single quantal responses to nerve stimulation were obtained by perfusing the preparation with saline solution containing $\frac{1}{3}$ normal Ca^{2+} and $6\times$ normal Mg^{2+} . Measurements of quantal size were corrected to correspond to the hyperpolarised level of membrane potential at which the e.p.s.p.s were measured; that is, m was determined from the relation: $\bar{v}_{\text{hyp}}/\bar{v}_{1\text{hyp}}$, where $\bar{v}_{1\text{hyp}} = \bar{v}_1(E_{\text{hyp}} - 15)/(E_r - 15)$. E_{hyp} is the membrane potential at which the e.p.s.p. was measured and E_r the resting membrane potential. The equation assumes the reversal potential for the e.p.s.p. is at -15 mV (refs 2 and 3). Input resistances (R) were determined routinely in all cells. The conductance (Δg) produced by a single quantum of ACh was calculated from the relation: $\Delta g = \bar{v}_1/R(E_r - 15)^4$.

Table 1 summarises the mean quantal contents of e.p.s.p.s in which the signal-to-noise ratio was high enough for the average quantal amplitude of the m.e.p.s.p.s to be estimated with an accuracy of $\pm 10\%$. The mean quantum contents of e.p.s.p.s recorded from axotomised cells were significantly lower than in control cells. The actual difference might be somewhat greater than indicated in Table 1 because of the underestimate of control e.p.s.p. amplitudes already mentioned in connection with Fig. 1b and c. The results summarised in Table 1 therefore confirm the observations obtained previously by more indirect measurements¹.

It could be argued that the lower quantum contents measured after axotomy were due to partial detachment of the presynaptic terminal from the postsynaptic cell so that the number of release sites apposed to the postsynaptic receptors was reduced. Detachment of presynaptic terminals after postsynaptic axo-

Table 1 Comparison of m of e.p.s.p. and of Δg produced by one quantum in control and axotomised cells

	$m \pm \text{s.d.}$	$\Delta g \pm \text{s.d.}$ ($\times 10^{-10}$ mho)	N
Control	82.4 ± 34.0	4.32 ± 3.41	12
Axotomised	40.6 ± 21.7	2.51 ± 1.11	10
P (t -test)	< 0.01	> 0.1	

N , number of experiments.

For unknown reasons, m in control cells is significantly higher and Δg lower than found by Martin and Pilar⁵.

tomy has indeed been found to be the major reason for loss of synaptic transmission in several preparations^{6,7}. In the ciliary ganglion of chickens, synapses are electrically coupled³ and detachment would be expected to reduce the coupling between presynaptic terminals and ganglion cells. The fact that the average amplitude of the coupling potentials was reduced after axotomy (6.6 mV compared with 18.3 mV in the control group) and that the percentage of cell profiles lacking synaptic contacts was increased¹, may therefore suggest partial detachment. As reported previously, however, the faster rate of fatigue of chemical transmission at synapses in axotomised preparations¹ cannot be explained on this basis. Moreover, in several axotomised cells, e.p.s.p.s smaller than ever observed in control cells were preceded by a coupling potential of normal amplitude (Fig. 1d). These findings suggest that the effects of axotomy on the parameters of synaptic transmission can occur before significant detachment. Similar observations have been made on axotomised cat spinal motoneurons⁸.

Table 1 also indicates that the unit conductance change, Δg , produced in the postsynaptic membrane by the interaction with one quantum of transmitter substance, was lower in axotomised than in control cells. The difference in Δg , however, is not statistically significant. In fact, two out of three of the axotomised cells had values of Δg larger than the smallest Δg observed in the control cells. This suggests that a decrease in transmitter release can occur before, or in the absence of, a reduction in postsynaptic ACh sensitivity. The lack of significant reduction in Δg is in contrast to the reduced sensitivity of the axotomised cells to iontophoretically applied ACh².

In the present experiments described here, however, the axotomised cells were selected for particularly high miniature potential amplitudes. Many of the cells impaled, particularly those with small e.p.s.p. amplitudes, could not be used for calculations of m because m.e.p.s.p.s were absent or too small to be measured accurately. Such cells had resting potentials and input resistances similar to those of cells listed in Table 1 and therefore would have been expected to produce measurable m.e.p.s.p.s if the underlying unit conductances had been the same. The disparity between the reduced sensitivity to exogenous ACh and lack of a marked reduction in Δg can also be explained in another way, namely by supposing that single receptor sites are lost after axotomy one at a time, together with the release sites apposed to them. This would lead to a decrease in overall ACh sensitivity with no reduction in the quantal conductance change, Δg , at the remaining sites. The reduction in quantum content would then be due, at least in part, to loss of release sites. Alternatively, postsynaptic receptor sites might disappear without accompanying loss of corresponding presynaptic release sites. Quantal release in such regions would then be ineffective and there would be an apparent reduction in m . In this case the increased susceptibility of the e.p.s.p. to fatigue during repetitive stimulation¹ would be the only electrophysiologically identifiable presynaptic consequence of postsynaptic axotomy. At present it is not possible to decide between these mechanisms.

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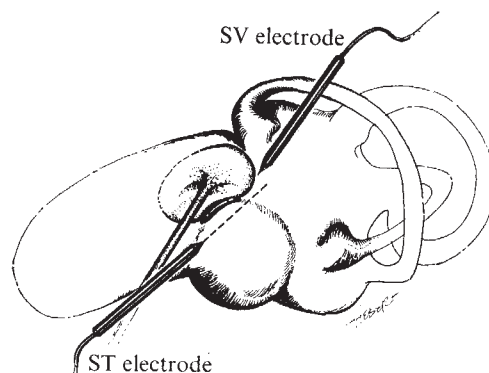
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Re-examination of avian cochlear potentials

THE aim of this study is to provide a basic description of the cochlear receptor potentials, the cochlear microphonic (CM) and the summing potential (SP) of the bird. Although several workers have examined these avian potentials in limited aspects, a comprehensive survey of their frequency and intensity behaviour is long overdue. Moreover, a re-examination of the avian potentials has seemed necessary because the potentials are reported to differ vastly from the mammalian CM and SP and one is left with the baffling implication that the middle and/or inner ears of these classes operate with a degree of dissimilarity exceeding what might reasonably be expected on the basis of structural difference. For example, the avian CM sensitivity function has been reported¹ to be flatter than the middle ear transfer function in a comparable species² thus implicating an unknown sort of cochlear element in the frequency response of the avian system. Second, the slopes of avian CM input-output functions have been reported as considerably less than unity and thus less steep than in the mammal^{1,3–7} leaving unanswered the question of whether the mechanics of the middle ear or basilar membrane, or peculiarities of the receptor cells *per se*, enforce the nonlinear intensity response in the bird. Third, if, as reported, the avian summing potential is appropriately described as a unitary negative response, manifest only at frequencies above 600 Hz (refs 4, 7–10), one can hardly reconcile this with the observation that the SP exists as both a positive and negative response in the mammal, and certainly occurs at even the lowest audible frequencies^{11,12} in that class. While each of the above differences implies a fundamental dichotomy between the avian and mammalian cochleae, such differences were not found in the present study. We did however use a new intracochlear electrode configuration in the elucidation of the parametric behaviour of the CM and SP in the pigeon. Fine tungsten electrodes, flame-sealed in capillary tubing, were placed in the basal scala vestibuli (SV) and the scala tympani (ST) proper. This electrode configuration made differential recordings possible and avoided electroanatomical effects, believed to be inherent in previous recessus tympani or round window recordings in the bird. The following differentially measured avian functions are discussed: the 1- μ V r.m.s. CM isopotential curve, CM isovelocity curves (relative to a constant footplate velocity of 10^{-6} mm s⁻¹), CM input-output functions, CM "degree of nonlinearity" functions and SP isovelocity functions. Our results show that these functions do not differ significantly from their mammalian counterparts.

Racing pigeons (*Columba livia*), generally less than three

Fig. 1 Illustration of electrode locations used in obtaining intracochlear differential recordings in a pigeon preparation. SV, scala vestibuli; ST, scala tympani.



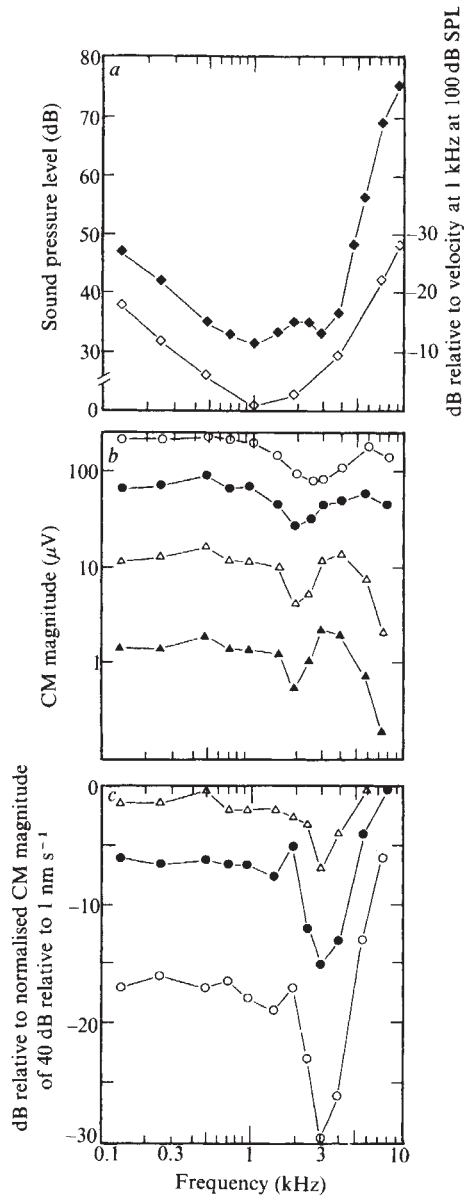


Fig. 2 *a*, Median, differentially recorded, 1- μ V r.m.s. CM isopotential curve from seven pigeons (left ordinate, $\blacklozenge$). For comparison (right ordinate, $\diamond$) is shown an inverted representation (see text) of an average middle ear transfer function measured by the Mössbauer technique in five Barbary doves². *b*, Differential CM isovelocivity functions from one pigeon. The parameter is footplate velocity in dB relative to 1 nm s⁻¹. *c*, "Degree of CM nonlinearity" exhibited by the pigeon shown in *b*. The deviations of the 60, 80 and 100 dB isovelocivity curves from their projected values, assuming a simple linear operation, are expressed in dB (see text). The parameter is footplate velocity in dB relative to 1 nm s⁻¹, $\circ$, 100 dB; $\bullet$, 80 dB; $\triangle$, 60 dB; $\blacktriangle$, 40 dB.

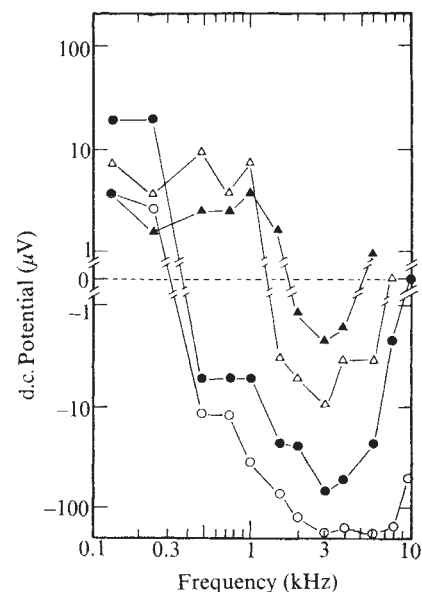
months old, were used. The birds were sedated with 20 mg kg⁻¹ pentobarbital 15 min before anaesthesia with 64 mg kg⁻¹ ketamine. Supplemental half-doses of ketamine were administered every 90 min thereafter. A tracheostomy was performed and the pigeons were respiration throughout surgery and the data collection period. The animals were maintained at 107 °F with a feedback-controlled heating pad. The surgical approach used was a dorsolateral one described by Wever and Bray¹. Figure 1 shows the electrode locations used. The wire of the SV electrode could be cut quite short since that scala is shallow in the bird^{13,14}. By contrast the wire of the ST electrode had to traverse the recessus tympani before the tip could be appropriately positioned for differential recordings. It was therefore necessary to fuse a 1.5–2.0-mm glass in-

sulating shoulder along the wire of that electrode. To assess whether damage occurred during the placement of this long electrode tip into the rather inaccessible scala tympani, the sensitivity of the CM and the magnitude of whole nerve action potential, both recorded from the SV electrode, were compared before and after the ST electrode was placed.

In Fig. 2*a*, a median, differentially measured 1- μ V CM isopotential curve (that is, sound pressure levels (SPL) required to produce 1- μ V r.m.s. CM) for 7 pigeons is presented in conjunction with a reciprocal form of an average middle ear transfer function measured in five Barbary doves². The inversion of that middle ear function was achieved by expressing footplate velocity measurements² in dB relative to the maximum velocity obtained at a constant SPL (that is velocity at 1,000 Hz). In Fig. 2*a*, it should be noted that the contours of the inverted middle ear transfer function and the CM isopotential function are well matched below 4,000 Hz. This agreement corresponds well with other studies in suggesting that the CM sensitivity in a normal cochlea is proportional to the footplate velocity^{15,18} up to a cutoff frequency that depends on electrode location. Moreover, the correspondence suggests that the influence of the middle ear on cochlear response could be minimised by referencing input levels to a constant footplate velocity. This has been shown to be the case in studies of mammalian cochlear function¹⁸. Consequently, in the parametric measurements being reported, the SPL at the eardrum was adjusted from frequency to frequency on the basis of the middle ear transfer function of the Barbary dove to produce an approximately constant footplate velocity across frequency. To this end a velocity of 10⁻⁶ mm s⁻¹ (1 nm s⁻¹) was arbitrary called 0 dB in the present study and corresponds approximately to a 20 dB SPL signal at 1,000 Hz.

Figure 2*b* shows differentially measured CM isovelocivity functions (that is, CM magnitude measured at constant footplate velocity) at 20-dB intervals in one pigeon. As expected using a constant footplate velocity, the functions are quite flat below the best frequency of the electrode pair. The notch seen at 2 kHz could be a consequence of the irregularity of the middle ear response (not manifested in the average transfer function) or a cochlear effect. Similar notches below the best frequency are also seen in guinea pig CM functions¹⁷. At the 40 dB level, the 3,000-Hz en-

Fig. 3 Differential SP isovelocivity functions from one pigeon. Using averaged responses, measurements were made 25 ms after stimulus onset. The parameter is footplate velocity level expressed in dB relative to 1 nm s⁻¹. Symbols as for Fig. 2.



hancement indicated this to be the best frequency of the differential electrode location used in this, and most (see Fig. 2a) pigeons. Were input output functions to be derived at low frequencies from the data shown in Fig. 2b, they would have an initial slope of 1.0 in double logarithmic coordinates. This indicates that the avian middle ear, the basilar membrane, and the contributing receptor cells have an operating range characterised by simple linearity over at least some portion of their dynamic range.

Figure 2c was derived from the data contained in Fig. 2b. In the construction of Fig. 2c, the response magnitude of the curves in 2b was normalised by shifting each curve vertically by an amount equivalent to the expected linear increase in the CM (commensurate with the increase in input level). The "degree of nonlinearity"¹⁸ functions shown in Fig. 2c were then derived by converting to dB the deviations of each normalised curve from the 40-dB isovelocity output. In Fig. 2c, it is seen that a region of pronounced nonlinearity develops around the best frequency. As the input level increases, the region of nonlinearity spreads to increasingly lower frequencies. The coincidence of the best frequency and the frequency of earliest and most pronounced nonlinearity is noteworthy inasmuch as CM nonlinearities are most prominent $\frac{1}{3}$ to $\frac{1}{2}$ octave above the best frequency in the guinea pig¹⁸. Nonlinearities at frequencies below the region of early nonlinearity, although clearly present, do not seem to be frequency-dependent in their severity. This is suggestive that two mechanisms produce CM nonlinearities; one mechanism being simply dependent on input level and one being also influenced by frequency.

The frequency response pattern of the differential component (SV-ST potential difference) of the summing potential is shown in Fig. 3. The earliest (40 dB level) and most pronounced negative component occurs at the best frequency (3,000 Hz) of the electrode pair. Similar to the summing potential in the mammal, the negative response band spreads to increasingly lower frequencies as the input level is increased. It is apparent, contrary to earlier reports, that there is a positive SP component at lower frequencies in the pigeon.

One may conclude that the salient properties of both receptor potential components (CM and SP) are very similar in representative avian and mammalian cochleas. It is likely that the differences shown between the results of the present and earlier work on avian receptor potentials are accountable by the generally excellent physiological condition of our animals, the lack of prolonged or excessive sound exposure in the present work, and by the electroanatomical differences between the present differential electrode location and the previously used round window or recessus tympani sites.

The only significant discrepancy found between avian and mammalian receptor potentials is that the CM nonlinearities and the most pronounced negative differential SP component (itself a nonlinear phenomenon) occur at the best frequency in the pigeon, whereas these nonlinearities occur above the best frequency in the guinea pig¹⁸. In both classes the occurrence of these nonlinearities seems to correspond to the relative frequency at which noise-induced hearing loss occurs. In the parakeet, such losses occur at the centre frequency of the eliciting noise¹⁹, while in the mammal, they appear at about $\frac{1}{2}$ octave above the centre frequency. A possible explanation, one suggested by Saunders and Dooling¹⁹, relates to the observation that travelling wave envelopes are sharper in the bird than in the mammal.

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Rod-cone mechanism underlying the Purkinje shift

TABLES have been published¹ of averaged brightness matches made by 16 observers who had compared 11 spectral lights (from 410 to 710 nm) with the CIE standard radiator in a 15° field set at various levels in the mesopic range. The scotopic and photopic luminances (S, P) of each light were calculated by multiplying the spectral radiant power for the match by the corresponding values of the CIE V' and $\bar{y}_{10}$ functions, with appropriate weighting factors. $\bar{y}_{10}$ was used because of the large field. The spectral data can readily be converted to the usual CIE system, and in fact the choice is immaterial in the following argument. As the matches were mesopic, neither S nor P alone can represent the luminance, and the Tables were used later² to define this quantity as a function of (S, P). Arithmetic manipulation of the data now suggests a simple rod-cone mechanism responsible for the sensation of brightness.

The first aim of my study was to refer the matches to an ideal light which would stimulate the cones preferentially. A deep red would fulfil this, as even at absolute threshold, redness is still visible³, indicating a considerable cone response compared with the rod response. The S value for 680 nm is only 1/70 of the P value (P_{680}), and this wavelength was selected as the reference. Light at 710 nm would also be suitable, but the data were deficient at higher levels.

For shorter wavelengths, the ratio S/P gradually increases to a limit of about 10/1 at 440 nm. To compare the various values of (S, P) with the reference, the P value of each light was subtracted from P_{680} and this showed that the difference, ΔP , depended only on S (Fig. 1).

Although derived from experiments done at seven levels spanning an enormous luminance range, most of the points lie on a straight line with a slope of about 0.5. The obvious conclusion is that

$$\Delta P \approx \sqrt{(M^*S)} \quad (1)$$

where M^* is some constant with the dimensions of luminance. Thus a light with scotopic and photopic luminances, S and P respectively, is equivalent in brightness to a red light at 680 nm of photopic luminance P_{680} , where

$$P_{680} \approx P + \sqrt{(M^*S)} \quad (2)$$

Some points stray far from the line, especially those for long wavelengths. Here ΔP is the difference between similar quantities and is thus bound to fluctuate widely. But a more subtle

explanation is required, because the points for 590 nm, 620 nm and 650 nm generally lie below the basic line of the graph (Fig. 1).

The points at variance can be brought into line, *ad hoc*, by reducing their S values, and to do this the CIE $V'\lambda$ curve from which S is calculated must also be reduced at long wavelengths. (Without some correction, equation (2) is bound to fail at 680 nm because the S contribution, although small, is finite.) More measurements are needed for an accurate estimation of the required factor, but inspection suggests a value of about 0.5 at 620 nm, dropping to about 0.25 beyond 680 nm. The equation could then be made to sum to a similar total for all lights of a given equivalent luminance, even for reds deeper than 680 nm, and it could be postulated to represent that combination of rod and cone responses which initiates the sensation of brightness, without reference to a standard.

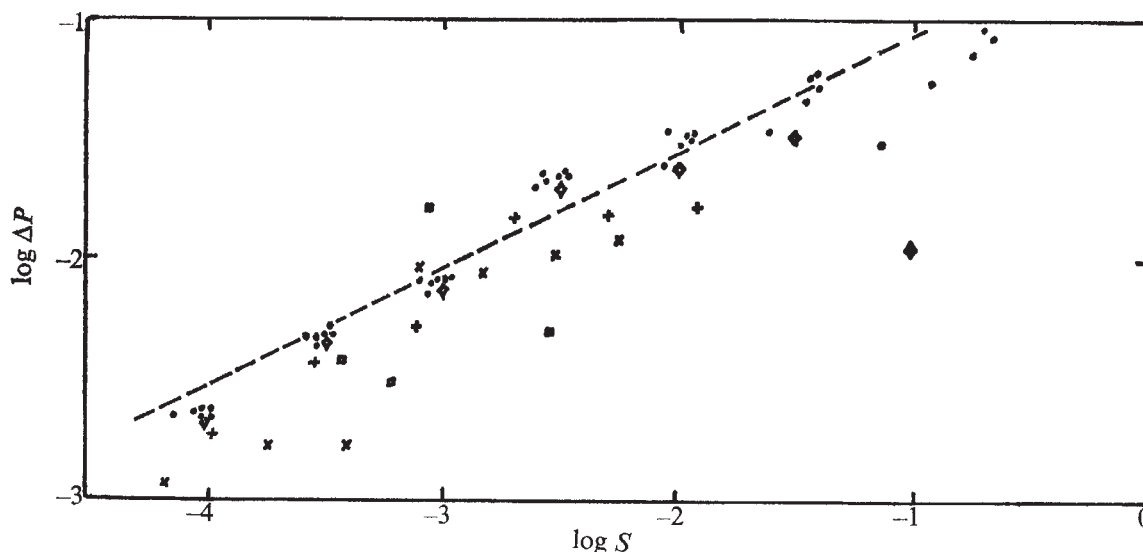


Fig. 1 Plot of ΔP against S on log scales. The quantities $\Delta P, S$ are in cd m^{-2} . +, 590 nm; $\times$, 620 nm; $\square$, 650 nm; $\bullet$, 410–560 nm; $\diamond$, standard radiator; — — —, equation (2) with $M^* = 0.08 \text{ cd m}^{-2}$.

The suggested modification to $V'\lambda$ is consistent with the initial assumption that, even at threshold, red lights still stimulate cone vision. The likely extrapolation of the graph in Fig. 1 is also intriguing in this respect, because if it continued at a slope of less than unity until it reached the threshold, which lies only one log unit below the lowest point, this would mean that the Purkinje shift is never completed.

For generality, the scheme must include non-monochromatic lights, which have been shown to behave photometrically like monochromatic lights with the same (S, P) coordinates^{1,4}. The special case of the standard radiator (a black body at 2,043 K) can be derived from the Tables because its luminance L_v is by definition identical to its S and P values, and can be subtracted from P_{880} to yield ΔP , which is then plotted against L_v ($\equiv S$).

The resultant points (Fig. 1, $\diamond$), follow the general trend through the mesopic range, except that the point at the highest level falls well below the graph. This apparently high discrepancy represents about 25% of P and is caused by additivity failure at high levels^{1,4}. The problem is not directly relevant here, where the rod-cone mechanism, and not the cone-cone systems, is being analysed. It may be concluded that equation (2) is valid to a good approximation for a variety of lights throughout the mesopic range.

This general relationship invokes several considerations. It suggests first, a simple mechanism underlying the Purkinje shift. As there are no cross-product terms, neither inhibition of the rods by the cones at high levels, nor inhibition of the cones by the rods at low levels, needs to be postulated. The systems

are independent, the rate of increase of the rod contribution diminishing progressively the higher the level. For this effect, a power function with an exponent less than unity is required, not necessarily exactly 0.5. Whether this represents any particular physiological feature in the retina or subsequent neural pathway is problematical, but at least one could search for a 'square-rooting' synapse involving the rod output.

Second, the formula suggests a simple mesopic photometer. This would require two photocells calibrated to the photopic and scotopic curves respectively, the scotopic signal being processed by a square-root circuit before addition to the photopic signal. Lights with the same luminance would therefore generate the same combined output in accordance with equation (2).

Third, intractable mesopic calculations are rendered relatively straightforward. The ideal reference stimulus may be used as a

dummy, to convert to any other reference light. For example, for the CIE standard radiator, $S \equiv P \equiv L_v$ by definition. Thus for a light with components (S, P) the simultaneous equations are

$$P_{880} = P + \sqrt{(M^*S)} \quad (3)$$

$$P_{880} = L_v + \sqrt{(M^*L_v)} \quad (4)$$

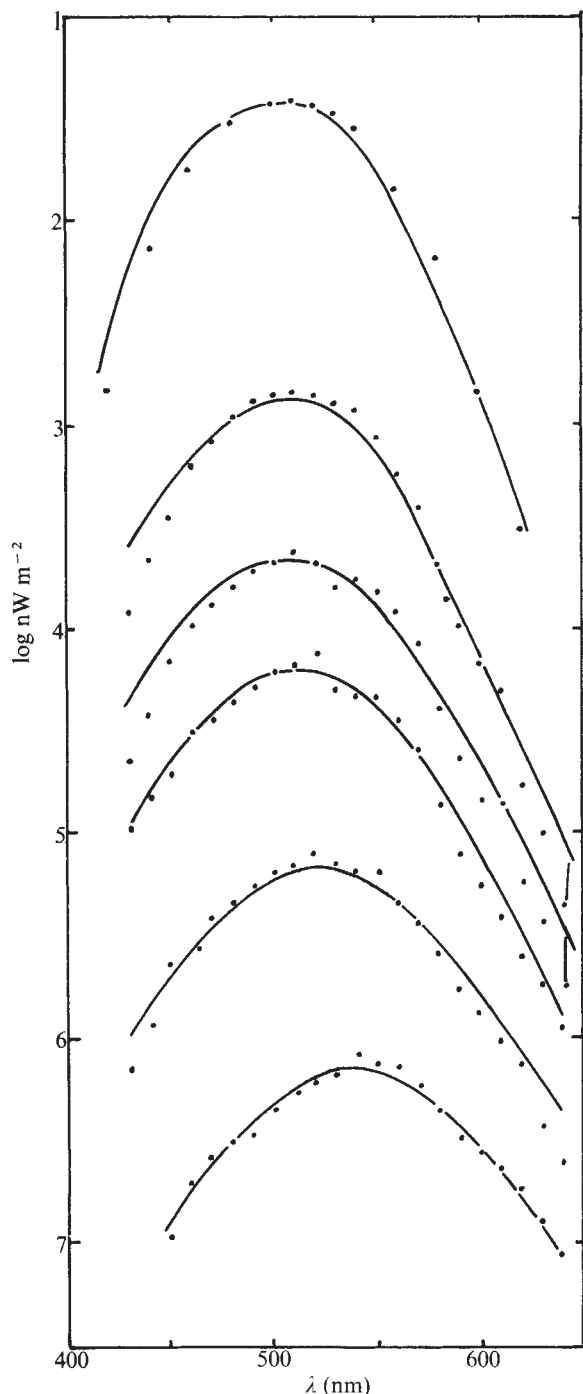
By elimination of P_{880} , L_v emerges as a quadratic solution in terms of S , P and M^* .

M^* is related to a parameter M , dependent on field size². A value of 0.06 cd m^{-2} has been suggested for a 15° field; a somewhat higher figure of about 0.08 cd m^{-2} is needed to fit the data in Fig. 1, if the discrepant long wavelength points are ignored.

By an extension of this mathematical analysis 'mesopic spectral visibility curves' can be produced. Although these are somewhat misleading⁴, since they cannot be used to calculate luminances from spectral power distributions, they are commonly used to display the results of spectral brightness matches. Observer D's results from Kinney's classic set⁵, measured in a 2° field at a peripheral angle of 10° , are shown in Fig. 2, together with examples of curves calculated using equation (2). The agreement is close, except that the smooth theoretical curves do not reproduce the bumps ascribed by Kinney to cone activity.

Evidently, much information about brightness matching in

Fig. 2 Comparison of Kinney's mesopic visibility curves for subject D with curves calculated from equation (2). The radiant power for a match is plotted in nW m^{-2} on an inverted log scale against the wavelength (λ) in nm.



the mesopic range can be derived from equation (2). Even its inadequacies suggest plausible modifications to the CIE V/λ curve, in order to represent the rod response to long wavelength light more accurately. I suggest therefore, that it expresses some fundamental rod-cone mechanism mediating the sensation of brightness.

I thank Dr Kinney for providing the data used in Fig. 2.

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Effects of enkephalin and morphine on Renshaw cells in feline spinal cord

It has been suggested that enkephalin is the endogenous ligand for the opiate receptor¹⁻³. This substance is unevenly distributed throughout the brain^{3,4} and occurs as two pentapeptides—met- and leu-enkephalin⁵. These substances have been shown to possess analgesic activity when injected intracerebroventricularly into rodents^{6,7} and to depress the firing rate of single neurones in the brainstem of cats⁸ and rats⁹. In the present experiments the effects of synthetic met-enkephalin have been compared with those of morphine on Renshaw cells in the feline spinal cord using the micro-electrophoretic technique. This cholinceptive interneurone is particularly suitable for such a comparison since it possesses stereospecific opiate receptor sites^{10,11}. The results obtained show that both met-enkephalin and morphine directly excite Renshaw cells and that these actions are antagonised by naloxone.

Experiments were carried out on four cats anaesthetised with pentobarbitone sodium (35 mg kg^{-1} intraperitoneally initially). Blood pressure and temperature were monitored continuously. The spinal cord was exposed by lumbar laminectomy and the central ends of S_1 and L_7 ventral roots were stimulated to identify Renshaw cells. Extracellular recordings were obtained with the centre barrel (4 M NaCl) of seven barrel microelectrodes (tip diameter $6 \mu\text{m}$). The outer barrels contained aqueous solutions of the drugs to be ejected electrophoretically; these were: morphine sulphate (0.05 M), naloxone hydrochloride (0.1 M), L-glutamate sodium (0.5 M), acetylcholine chloride (0.5 M), glycine (0.5 M, pH 3) and met-enkephalin (0.018 M, pH 4). Action potentials were recorded and displayed using conventional techniques.

The effects of electrophoretic ejections of met-enkephalin were compared with those of morphine on 14 Renshaw cells. Met-enkephalin (50–80 nA for 15–40 s) ejected as a cation, directly excited eight neurones. The onset of and recovery from the excitatory action of met-enkephalin was rapid (3–8 s) and similar to that observed with morphine (50–100 nA), which directly excited eleven neurones including the eight excited by met-enkephalin (Fig. 1a). Desensitisation to the excitatory effects of met-enkephalin were not observed on repeated ejection of this substance. Compared with morphine, met-enkephalin was approximately equipotent as an excitant on the basis of equieffective ejecting currents (Fig. 1a).

The specific opiate antagonist, naloxone, has previously been shown to antagonise the excitatory effects of morphine and acetylcholine but not those of excitatory amino acids on Renshaw cells¹⁰⁻¹². This effect of naloxone was also demonstrated in the present experiment (five tests on three cells). In addition, Figure 1a shows that naloxone (30 nA for 3–4 min) reversibly antagonised the excitatory effects of met-enkephalin. Similar effects were obtained in six tests on four more Renshaw cells.

Neither met-enkephalin nor morphine directly depressed the firing rate of any of the neurones studied in this investigation. When met-enkephalin (40–80 nA) was ejected for prolonged periods (2 min or more) however, the sensitivity of neurones to acetylcholine was decreased. This effect of met-enkephalin was observed on each of eight neurones, four of which were directly excited by a previous brief ejection of met-enkephalin. In contrast, prolonged ejections

of morphine (25–40 nA) enhanced the excitatory effects of acetylcholine (five cells) and L-glutamate (two cells) in agreement with previous findings¹⁰. The reduction in acetylcholine sensitivity seemed to be a selective action of met-enkephalin in so far as the excitatory effects of morphine and L-glutamate were either unaffected (three neurones) or only slightly reduced (two neurones). Furthermore, the sensitivity of neurones to both morphine and amino acids recovered immediately on terminating the ejection of met-enkephalin, whereas recovery of the acetylcholine sensitivity was often prolonged, requiring up to 5 min or more. The effects of met-enkephalin on the acetylcholine- and morphine-induced excitation is illustrated in Fig. 1b.

Met-enkephalin (40–100 nA) had no obvious effect on the depressant action of glycine in tests on five neurones whereas morphine (25–40 nA) reversibly reduced this action of glycine in tests on the same neurones. This action of morphine reported previously is not antagonised by naloxone^{12,13}.

In the present study it was significant that the only effect of met-enkephalin in common with that of morphine was an ability to excite Renshaw cells, an action which was also antagonised by naloxone. The excitatory action of morphine has previously been shown to be stereospecific at this site^{11,12}. The present experiments therefore provide direct evidence that met-enkephalin acts on a central opiate receptor and supports the notion that enkephalin is the endogenous ligand for the stereospecific opiate receptor¹.

The enhancement of the excitatory responses to acetylcholine and amino acids excitants by morphine has been considered to be related to the excitatory properties of morphine on cholinergic neurones¹². The apparent selective reduction in neuronal sensitivity to acetylcholine produced by met-enkephalin was therefore unexpected, since this substance also directly excited cholinergic neurones. The reasons for these different actions of met-enkephalin and morphine are not known. Note, however, that the active opiate levorphanol also causes a prolonged reduction in acetylcholine sensitivity of Renshaw cells¹².

Thus the present experiments indicate that enkephalin has complex actions on a central cholinergic neurone, including a specific opiate type effect. Preliminary results obtained on non-cholinergic interneurons suggest that the effects of met-enkephalin described here are confined to cholinergic neurones. More extensive experiments are

necessary, however, to verify this point and to establish a possible physiological role for endogenous enkephalin.

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Depression of nociceptive and other neurones in the brain by iontophoretically applied met-enkephalin

It has been suggested that enkephalin might act as a neurotransmitter or neuromodulator in the mammalian brain¹, and recent experiments^{2,3} have shown that microiontophoretic application of enkephalin will depress brainstem neurones in both rat and cat. We have confirmed these observations on neurones in other regions of the rat brain and have extended the study to an investigation of the action of iontophoretic met-enkephalin on neurones activated by painful stimuli.

Experiments were carried out on adult male rats anaesthetised with 1% halothane in O₂ and prepared for single neurone recording from the cerebral cortex and thalamus (12 rats) or dorsal medulla (4 rats). Conventional techniques were used for single neurone recording and the iontophoretic application of drugs⁴. Five-barrelled micropipettes (tip-size about 6 μ m) were used in most experiments with the outer barrels filled with solutions of L-glutamate (0.2 M, pH 8), GABA (0.2 M, pH 3.5), glycine (0.2 M, pH 3.5) and met-enkephalin (5 mg ml⁻¹, that is, about 8 mM in either 200 mM NaCl or in water; no adjustment of pH). In 7 of the 12 experiments some microelectrodes were double-barrelled and the only drug tested was met-enkephalin. NaCl 4 M was used in the recording barrel of all electrodes.

In a preliminary survey, in the cerebral cortex, thalamus and dorsal medulla, met-enkephalin applied with positive (0–400 nA), but not negative current, was found to inhibit 59 out of 88 neurones considered to be adequately tested. On 4 of the 88 neurones the action of enkephalin seemed to be excitatory. Appropriate controls to exclude current artefacts were carried out. The action of short applications (< 60 s) of met-enkephalin was uniformly rapid in onset and offset, typically taking less than 10 s to develop and this is similar to the time course of GABA's action in our experimental conditions, although extended application of enkephalin had effects which persisted for several minutes (Fig. 1). Desensitisation to the depressant actions of met-enkephalin was not observed when repeated short applications at intervals of 90–120 s were made to many neurones, sometimes over a period of 1 h or more. This is in contrast

Fig. 1 Ratemeter record. *a*, Excitatory effects of acetylcholine (ACh, 1 nA); met-enkephalin (ME, 50 nA) and morphine (M, 50 nA), and antagonism of these effects by naloxone (NX, 30 nA). *b*, Effects of a prolonged ejection of met-enkephalin on the excitatory responses induced by morphine (40 nA) and acetylcholine (6 nA). Both records are from the same Renshaw cell.

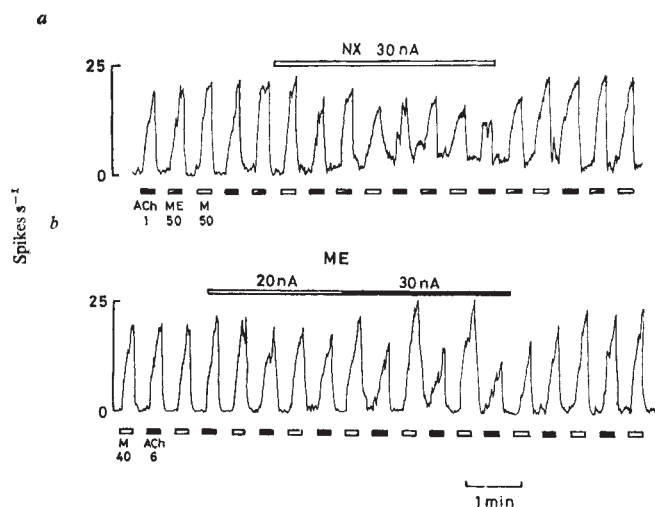
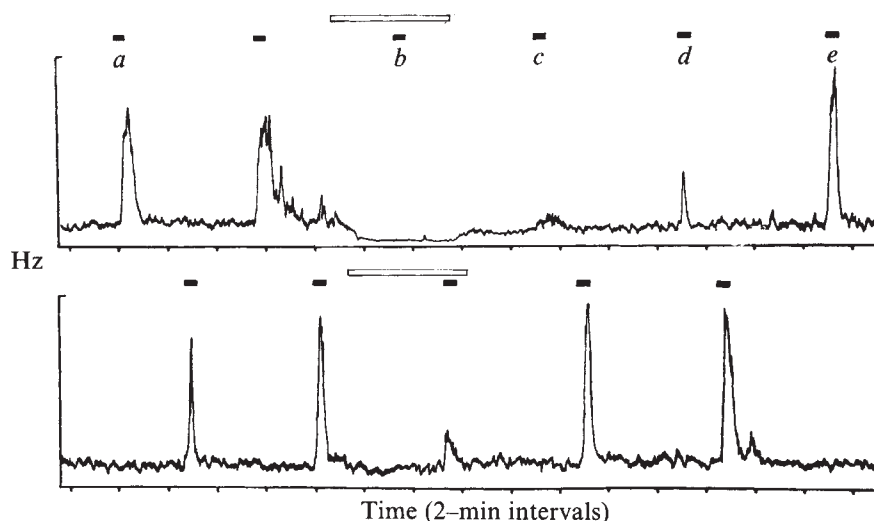


Fig. 1 Analogue ratemeter record of spontaneous firing of a nociceptive neurone (located at a depth of 4,430 μm below the cortical surface) in the rat thalamus. Lower section continuous with upper section. Short horizontal filled bars above record indicate immersion of rat's tail in water at 50 °C for 30 s; this treatment resulted in a brisk discharge of the neurone. For the duration of the first open bar over the record met-enkephalin was applied to the neurone with a current of 100 nA; this completely depressed the spontaneous firing and abolished the response to tail warming. The response to hot water was substantially recovered 16 min after removing the met-enkephalin. The second open bar above the record indicates removal of a 10 nA negative retaining current from the met-enkephalin barrel; although this had no detectable effect on the spontaneous firing rate, it markedly reduced the response to tail warming. The response was fully recovered within 5 min. The firing of this neurone was also depressed by GABA at 50 nA and the response to tail warming was partially blocked by GABA at 10 nA (not shown). *a-e*, Photographic records from the same experiment shown in Fig. 2.



to the action of morphine, in which case desensitisation has been reported after iontophoretic application to central neurones^{5,6}, but is consistent with the lack of desensitisation to the effects of enkephalin on peripheral tissues¹.

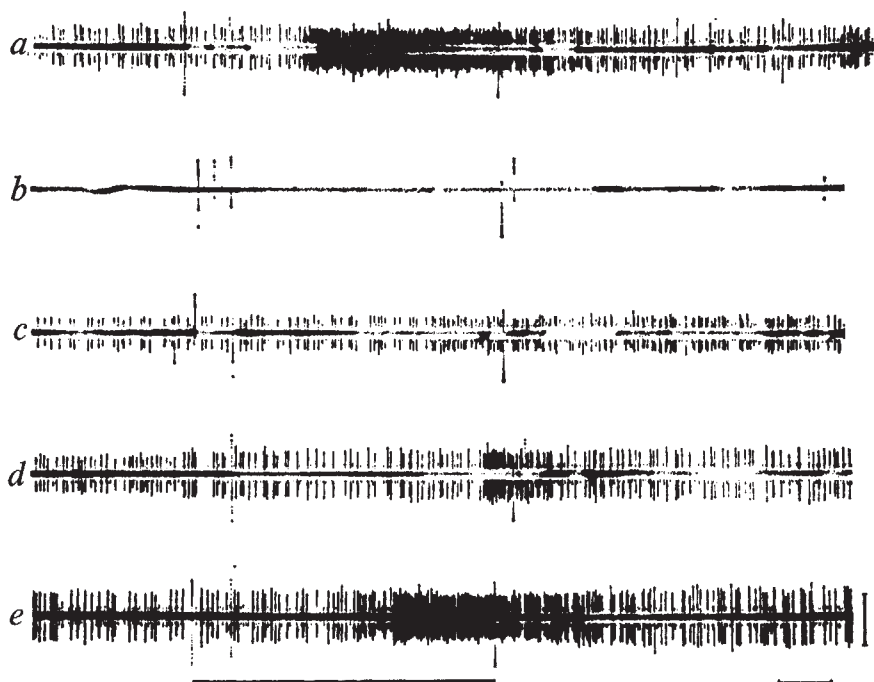
Met-enkephalin has also been tested on thalamic neurones thought to be excited by noxious stimuli^{7,8}. The position of the micropipette in the thalamus at the stereotaxic coordinates AP+3, L3, H4 to 7 (.. . 9) was confirmed both histologically and by recording field potentials^{10,11} evoked by electrical stimulation of the limbs and tail. In the present study neurones excited by either a strong tail pinch with a thermometer clamp or immersion of the tail in hot (50 °C) water were considered to be nociceptive in nature. Such nociceptive neurones were very sensitive to met-enkephalin, and this observation is supported by the reduction in the evoked excitation shown in the latter part of Fig. 1 produced simply by removing the 10-nA retaining current from the met-enkephalin-containing barrel of the pipette. The photographic traces in Fig. 2 show that high currents (50–100 nA) of met-enkephalin completely abolished both the

background spontaneous activity of this neurone and its response to noxious stimulation. This reduction in the response to noxious stimulation persisted for at least 12 min after the end of the met-enkephalin application (Figs 1 and 2). On seven other nociceptive neurones similar responses to met-enkephalin were obtained.

On eight unidentified neurones tested the depressant action of enkephalin was antagonised by intravenous naloxone (80–320 μg) on only two. This finding in the central nervous system is consistent with the results of experiments on isolated peripheral tissues³, in which it was discovered that the depressant action of enkephalin was less sensitive to reversal by naloxone than was the similar action of morphine.

The implications of the observations reported here can only be fully explored by further experiments, but it is evident that met-enkephalin will depress many neurones in the mammalian brain and, most interestingly, those located in the thalamus and which are excited by noxious inputs. The observations of Hughes¹² and others¹³ that enke-

Fig. 2 Continuous photographic records of the firing of the same nociceptive neurone illustrated in Fig. 1. *a-e*, Similarly marked ratemeter traces in Fig. 1. Each vertical deflection on the filmed record represents a burst of 4 or 5 action potentials, the larger deflections visible on some records (for example, *b*) are artefacts caused by touching the animal to apply a heat stimulus. The solid black bar beneath the records indicates the 30-s period over which the animal's tail was immersed in hot (50 °C) water. This produced a sharp increase in firing shown by the increase in density of record *a*. Before record *b*, met-enkephalin was applied at a current of 100 nA and completely abolished the spontaneous firing of the neurone and its response to tail warming. Before record *c*, this met-enkephalin application was terminated; although spontaneous firing had returned, the response to tail warming was still virtually absent. Records *d* and *e* illustrate the gradual recovery of the response to tail warming over the next 12 min. Horizontal bar, 5 s; vertical bar, 0.4 mV.



phalin is found in many areas of the brain not yet implicated in the organism's responses to pain, suggests that this substance may be an important inhibitory transmitter candidate at both nociceptive and other sites.

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Changes in amino acid sensitivity during polypeptide 'desensitisation'

CURRENT interest in polypeptides as neurotransmitter substances in the central nervous system (CNS) has been focused on their ability to depolarise motoneurons¹ and to excite single cells in various parts of the brain^{2–6}. An additional feature of their actions on nervous tissue, however, is the development of 'tachyphylaxis' or 'desensitisation' to their effects during repeated administration. This phenomenon has been reported with two structurally related undecapeptides in particular, substance P and eleidoisin, both in spinal cord⁷ and in brain^{3–5}. We report here that in the substantia nigra additional changes in amino acid sensitivity occur during such a period of desensitisation to eleidoisin.

Albino rats (220–270 g), anaesthetised with urethane

1.4 g kg⁻¹, were used during this study. Multibarrelled glass micropipettes (tip diameter 6 μ m) were used to record action potentials extracellularly and to administer substances by electrophoresis on to spontaneously firing neurones in the substantia nigra. Micropipettes were filled immediately before use by a glass-fibre method or by centrifugation. The recording barrel contained 3 M sodium chloride and another containing 1 M sodium chloride was used to balance the net current at the electrode tip to zero or to test for electrophoretic current effects. Other barrels contained eleidoisin dihydrochloride (Calbiochem) 24 mM, pH 4.5; sodium glutamate 0.2 M, pH 7.2; GABA 0.2 M, pH 3.5; glycine 0.2 M, pH 3.5; taurine 0.2 M, pH 4.0; and acetylcholine chloride 0.5 M pH 4.5.

Action potentials were recorded and displayed using conventional techniques. Substantia nigra neurones were identified histologically after pontamine-blue dye ejection or by their response after stimulation of the ipsilateral caudate nucleus⁸.

Short lasting application (30–60 s) of eleidoisin ejected as a cation (50–100 nA; mean 77.3 nA) excited the majority of substantia nigra neurones tested (50 out of 55 cells). One neurone was depressed. The time course of these effects was similar to that described previously⁵. When brief applications of eleidoisin were repeated at short intervals the excitatory response became progressively smaller until it could no longer be distinguished from the spontaneous activity. A progressive reduction in the excitation also occurred when a single administration of eleidoisin with rather less current (25–75 nA; mean 45.6 nA) was prolonged for 3.5–17 min.

Prolonged applications of eleidoisin also reversibly reduced the inhibitory responses to GABA (11 out of 20 cells), glycine (5 out of 13 cells) and taurine (9 out of 20 cells). Such effects, however, were not always observed on the same neurones, since glycine or GABA responses could be depressed without changes in taurine sensitivity occurring (Fig. 1); or taurine responses were depressed without any significant changes in GABA sensitivity. In only 4 of the 13 cells tested with all three inhibitory amino acids, were the responses to each compound entirely unaffected. In addition the excitatory effects of glutamate could be reversibly reduced (10 out of 11 cells), although on one occasion glutamate excitation was potentiated. Acetylcholine excitation was also reduced (2 out of 2 cells), although relatively fewer neurones were tested. These interactions during prolonged applications of eleidoisin were not usually accompanied by changes in background firing rate; neither

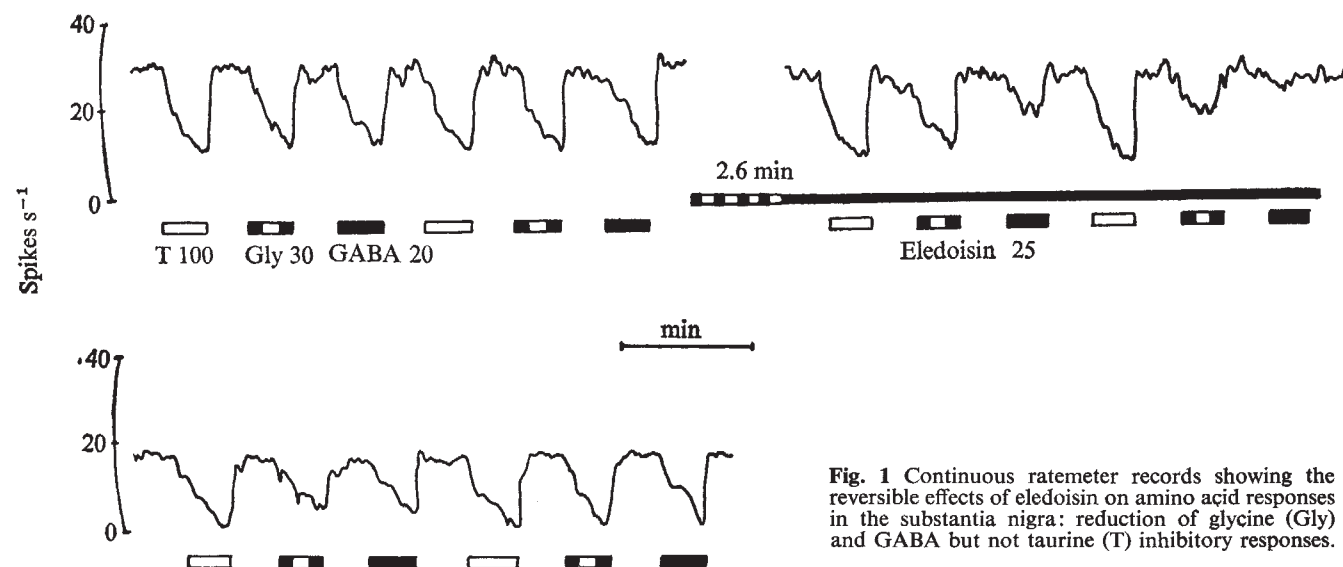


Fig. 1 Continuous ratemeter records showing the reversible effects of eleidoisin on amino acid responses in the substantia nigra: reduction of glycine (Gly) and GABA but not taurine (T) inhibitory responses.

were there any alterations in neuronal spike size, although occasionally this was slightly decreased.

Recovery of the responses to amino acids, after termination of eledoisin administration sometimes occurred within about 5 min, but more commonly was considerably prolonged (up to 25.6 min). After recovery of neuronal sensitivity to these agonists, however, a repeated administration of eledoisin produced the same pattern of interactions as observed previously. This suggested that on individual cells the observed interactions were reproducible and could indeed be selective.

Our results, in agreement with previous observations⁸, show that substantia nigra neurones are more sensitive to exogenously administered GABA than glycine and are more sensitive to glutamate than acetylcholine. Taurine, which occurs in significant amount in the substantia nigra⁹, also exhibits significant inhibitory actions, although it is less potent than either GABA or glycine. In addition the undecapeptide eledoisin excited most substantia nigra neurones, confirming our previous observations⁸. Moreover, repeated or prolonged administration of eledoisin produced desensitisation. During such a period of desensitisation the sensitivity of neurones to exogenously applied GABA, glycine, taurine, glutamate or acetylcholine was reduced. Overall, eledoisin reduced neuronal sensitivity to inhibitory compounds less consistently than to excitatory ones. It is not clear why selectivity to one or more neurally active substance could be observed on some neurones but not others. Note, however, that a reduction in the sensitivity of central neurones to exogenously administered glutamate or acetylcholine occurs after administration of the naturally occurring and structurally related undecapeptide, substance P (ref. 4).

Attention has been drawn to the potent excitant actions of polypeptides in the spinal cord¹ and in other areas of the brain^{2-4,6} but it is possible that such effects may be an artefact resulting from the exogenous administration of non-physiological concentrations of these substances. Many active polypeptides including eledoisin and substance P have a common C-terminal amino acid sequence¹. This property may be related to their depolarising properties as well as to the production of desensitisation and the modification of endogenous neurotransmission.

Although little is known concerning the precise mechanism of action or physiological role of polypeptides in the CNS their excitation of single neurones may be related to a neurotransmitter function. The modification of neuronal sensitivity to other putative neurotransmitters, however, suggests a more complex function which requires further elucidation.

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Hypothermia and intolerance to cold induced by intracisternal administration of the hypothalamic peptide neurotensin

NEUROTENSIN is a tridecapeptide (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-COOH)¹ isolated from bovine hypothalami². Using a sensitive and specific radio-immunoassay, Carraway and Leeman have demonstrated immunoreactive neurotensin in hypothalamic extracts of rat, mouse, rabbit and human tissue³. Intravenous (i.v.) administration of neurotensin to rats has been reported to cause hypotension (minimum effective dose 100 pmol kg⁻¹ = 0.167 µg kg⁻¹), cyanosis (minimum effective dose 1 nmol kg⁻¹ = 1.67 µg kg⁻¹)², and hyperglycaemia (minimum effective dose 200 pmol kg⁻¹ = 0.33 µg kg⁻¹)⁴. These effects are not prevented by adrenalectomy or hypophysectomy. The hypotensive effects are not altered by α- or β-adrenergic blockers. Neurotensin also stimulates the contraction of guinea pig ileum while relaxing rat duodenum *in vitro*. Neurotensin is as potent as bradykinin in its effects on guinea pig ileum and rat duodenum and thus is classified as a kinin². It increases plasma gonadotropin levels in ovariectomised, oestrogen-progesterone-treated rats after intravenous injection (minimum effective dose 5 nmol kg⁻¹ = 8.4 µg kg⁻¹)⁵. Intravenous administration of a dose of 3 µg kg⁻¹ to rats produces hypoinsulinaemia, hyperglycaemia and hyperglucagonaemia⁶.

Intracisternally (i.c.) administered thyrotropin-releasing hormone (TRH, effective dose 10 µg) and TRH analogues antagonise the depressant and hypothermic properties of various barbiturates⁷⁻⁹ and ethanol¹⁰. Neurotensin, administered i.c. but not peripherally, potentiates markedly the sedative effects of pentobarbital in the mouse¹¹. To understand this apparent synergistic interaction with pentobarbital further, we have

Fig. 1 Effect of i.c. neurotensin on the core temperature of mice subjected to a cold environment (4 °C). Data are expressed as °C ± s.e.m. Experimental values were compared with those of controls by Student's *t*-test (two-tailed). Small open circles, 0.1 µg of neurotensin (*n* = 11); small closed squares, Saline (*n* = 22); closed circles, 0.030 µg of neurotensin (*n* = 11); closed triangles, 1 µg of neurotensin (*n* = 121); small open squares, 0.3 µg of neurotensin (*n* = 6); large open circles, 3 µg of neurotensin (*n* = 6); large closed squares, 10 µg of neurotensin (*n* = 6); open triangles, 30 µg of neurotensin (*n* = 6). †*P* < 0.001; ††*P* < 0.01; †††*P* < 0.05.

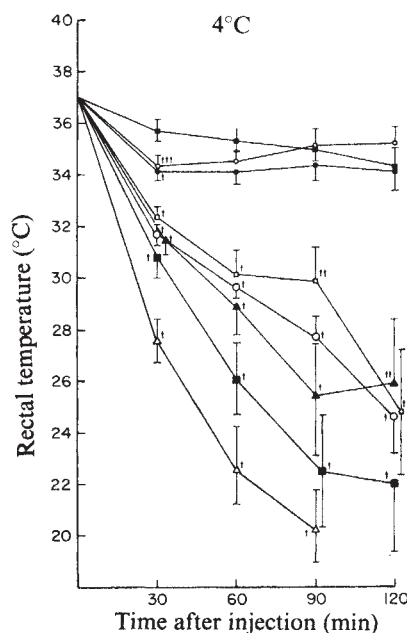


Table 1 Effect of intracisternal neurotensin on temperature regulation at 25 °C in the mouse

Substance	0 min	Rectal temperature (°C) ± s.e.m.			
		30 min	60 min	90 min	120 min
Saline (<i>n</i> = 7)	37.6 ± 0.184	38.1 ± 0.134	37.9 ± 0.315	37.7 ± 0.358	37.6 ± 0.280
Neurotensin 10 µg (<i>n</i> = 7)	37.8 ± 0.275	34.8 ± 0.253*	35.3 ± 0.155*	35.8 ± 0.193†	35.6 ± 0.420
Neurotensin 30 µg (<i>n</i> = 7)	37.7 ± 0.180	34.5 ± 0.421*	34.7 ± 0.506*	34.9 ± 0.641†	35.7 ± 0.476†

**P* < 0.001 by *t* test.†*P* < 0.01 by *t* test.

investigated the effects of centrally administered neurotensin on thermoregulation.

Adult male Swiss-Webster mice (24–25 g) were anaesthetised lightly with ether and injected i.c. with neurotensin (0.03–30 µg in 10 µl of vehicle) or 10 µl of vehicle (0.9% NaCl solution). Recovery from anaesthesia required about 1 min. Mice were then placed in individual plastic cages without bedding in a room maintained at 4 °C or room temperature (25 °C). Rectal temperatures were assessed at 30-min intervals with a thermistor probe. TRH, luteinising hormone-releasing hormone (LHRH), melanocyte-stimulating hormone-release-inhibiting hormone (MIF I), substance P and somatotropin release-inhibiting hormone (SRIF) were also tested at 4 °C by intracisternal injection in doses equimolar to 30 µg of neurotensin. Neurotensin was also injected i.v. at 10, 30 and 100 µg kg⁻¹. Those doses were substantially greater than those reported

administration of TRH has been reported to induce hyperthermia in rabbits¹², while others have shown that intracerebroventricular TRH induces hypothermia in cats¹³. Both the hypothermia at 25 °C and the considerable changes in cold tolerance at 4 °C observed after the i.c. administration of neurotensin suggest that it is involved in central thermoregulatory processes. This is also suggested by its only reported endogenous location, the hypothalamus, long implicated in control of temperature. It is premature to hypothesise a mechanism or locus of action of neurotensin in cold tolerance. The fact that central administration of this peptide has marked effects on both maintenance of body temperature and the duration of pentobarbital-induced sedation¹¹ indicates that it can affect the central nervous system directly. This is supported by lack of action of this peptide in both these situations after peripheral administration. The failure to find activity in these

Table 2 Effect of i.c. hypothalamic peptides on cold-induced (4 °C) hypothermia in the mouse

Substance*	0 min	Rectal temperature (°C) ± s.e.m.			
		30 min	60 min	90 min	120 min
Saline (<i>n</i> = 12)	37.2 ± 0.390	36.4 ± 0.476	36.0 ± 0.540	35.4 ± 0.550	35.3 ± 0.546
TRH (<i>n</i> = 6)	37.1 ± 0.177	34.4 ± 0.929	34.2 ± 0.947	34.3 ± 0.757	33.7 ± 1.22
LHRH (<i>n</i> = 6)	37.9 ± 0.196	35.2 ± 0.508	34.8 ± 0.734	34.0 ± 1.12	33.3 ± 1.40
MIF I (<i>n</i> = 6)	37.4 ± 0.095	36.2 ± 0.522	35.4 ± 0.876	35.2 ± 1.19	34.3 ± 1.55
Substance P (<i>n</i> = 6)	37.6 ± 0.236	36.8 ± 0.395	36.2 ± 0.585	35.2 ± 0.843	34.0 ± 1.57
SRIF (<i>n</i> = 6)	37.4 ± 0.293	36.0 ± 0.777	36.2 ± 0.650	34.6 ± 0.852	33.6 ± 1.44

*Doses equimolar to 30 µg of neurotensin in 10 µl of vehicle (0.9% NaCl).

to cause peripheral vascular changes and were administered to assess the possibility that such changes contribute to cold-induced hypothermia. Differences in rectal temperatures between control and experimental groups were analysed for statistical significance by Student's *t*-test (two-tailed).

Neurotensin given i.c. to mice in a cold environment markedly accentuated the decline in core temperature over 120 min (Fig. 1). This effect was dose related; the minimum dose showing an effect at 30 min was 0.03 µg. The i.c. administration of 10 and 30 µg of neurotensin also caused a significant decrease in core temperature at 25 °C (Table 1). The intracisternal delivery of TRH, LHRH, MIF, substance P and SRIF failed to produce any significant changes in rectal temperature from the control values at 4 °C during the 2-h period of observation (Table 2). In contrast to the action of neurotensin after intracisternal delivery no significant effect on cold tolerance was observed after intravenous administration of neurotensin (Table 3).

TRH is the only peptide previously reported to affect thermoregulation. Intracerebroventricular and intravenous

tests after peripheral administration of substantially greater amounts than are active centrally suggests the site of action of neurotensin lies within the blood-brain barrier. But the possibility that neurotensin i.c. exerts its effects by acting directly on cerebral vasculature or on central mechanisms that control peripheral vasomotor changes cannot be excluded. It is interesting that substance P, an undecapeptide that also induces hypotension after intravenous administration^{14,15}, has no effect on thermoregulation in a cold environment after central administration. In a similar manner, SRIF, a tetradecapeptide, failed to affect cold tolerance significantly. These findings suggest specificity of the neurotensin effect we have described.

In our experiments with mice, we found that even i.v. neurotensin at 100 µg kg⁻¹ failed to induce cold intolerance. On the other hand, Carraway and Leeman found in the rat that less than 2 µg kg⁻¹ given intravenously produces cyanosis and hypotension². We did not measure possible blood pressure changes in our mice, but constant observation revealed no cyanosis. Species differences may account for this discrepancy.

Table 3 Effect of intravenous neurotensin on cold-induced (4 °C) hypothermia in the mouse

Substance	0 min	Rectal temperature (°C) ± s.e.m.			
		30 min	60 min	90 min	120 min
Saline (<i>n</i> = 16)	38.6 ± 0.172	37.1 ± 0.191	36.8 ± 0.242	36.4 ± 0.358	35.8 ± 0.522
Neurotensin 10 µg kg ⁻¹ (<i>n</i> = 8)	37.8 ± 0.213	37.0 ± 0.245	36.8 ± 0.281	36.7 ± 0.313	36.3 ± 0.384
Neurotensin 30 µg kg ⁻¹ (<i>n</i> = 8)	39.0 ± 0.152	37.3 ± 0.243	37.1 ± 0.543	36.0 ± 1.22	35.4 ± 1.14
Neurotensin 100 µg kg ⁻¹ (<i>n</i> = 8)	38.8 ± 0.137	37.4 ± 0.195	37.3 ± 0.177	36.8 ± 0.205	36.5 ± 0.288

Species differences may also explain the null effect of TRH in our study, which contrasts with its reported effects on thermoregulation in rabbit¹² and cat¹³.

In the light of the evidence presented here, neurotensin can apparently be included in the growing list of peptides which seem directly to influence central nervous system function¹⁶.

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Pancreatic gastrin in foetal and neonatal rats

It has been suggested^{1,2} that pancreatic gastrin is stored in islet A₁ (or D) cells. There are, however, conspicuous histological and histochemical differences between antral gastrin cells and A₁ cells, indicating the storage of different peptide molecules in these two types of cell^{3,4}. Furthermore, pancreatic tumours producing gastrin are composed of cells that have no features in common with A₁ cells but often resemble antral gastrin cells^{5,6}. We have now tested a large number of gastrin antisera, most of which were specific for the C-terminal, bioactive, sequence of gastrin-17, on both unfixed and formaldehyde-fixed tissue, and found that all antisera reacted with antral gastrin cells but not with A₁ cells. Also, radioimmunoassayable pancreatic gastrin occurs at levels far below those expected if the A₁ cells stored gastrin⁷. We conclude that if gastrin-like peptides occur in the pancreatic A₁ cells they must be devoid of the bioactive, C-terminal sequence. While studying the ontogeny of the rat gastrin cell we have, however, found, at a certain stage of development, true gastrin cells in the pancreas.

In the pancreas, gastrin-immunoreactive cells were observed at day 18 of foetal life (the earliest stage studied). The cells occurred both in islets and scattered among exocrine cells (Fig. 1a). The gastrin cell number increased up to day 4 after birth, when it slowly began to decrease. A few pancreatic gastrin cells were seen in 12- to 20-d-old rats but at this stage they were usually restricted to the ductal epithelium. In rats older than 20 d gastrin cells were never observed in the pancreas (except for occasional cells in the epithelium of large excretory ducts). During all stages

the gastrin cells seemed uniformly distributed between the tail and the duodenal lobe.

In the duodenum of adult rats gastrin cells were scarce¹¹. The duodenum of rat foetuses contained, however, a fair population that could be detected at a foetal age of 18 d. Like the pancreatic gastrin cells, these cells increased in number until a few days after birth when their number (per unit area) started to decrease. In the antral mucosa, gastrin cells could be detected only after birth¹¹. Cells, with an ultrastructure indistinguishable from that of the presumed gastrin cells (G cells) of rat antral mucosa, were detected in antral and duodenal mucosa and in pancreas of neonatal rats (4-d-old) (Fig. 1b). The distribution pattern and number of G cells was in good agreement with that of the gastrin-immunoreactive cells. Cells with an ultrastructure reminiscent of G cells have previously been detected in cultures of neonatal rat pancreas^{12,13}. Such cells were not found in

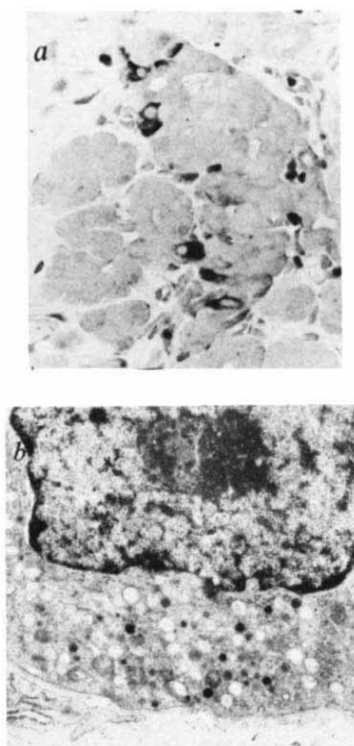


Fig. 1 a, Pancreas from 19-d-old rat foetus, showing an islet containing several peripheral gastrin cells (immunoperoxidase staining; $\times 217$). b, Pancreas from 4-d-old rat, showing a typical G cell (glutaraldehyde-fixed, osmicated specimen; $\times 7,800$). Foetal rats were removed at 18, 20 and 21 d of gestation, and newborn and young rats taken 1-30 d after birth. Adult male and female rats (200-300 g) were also used. After diethyl ether anaesthesia the antropyloric region, the duodenum and the duodenal lobe (that is, the portion of the pancreas that is adjacent to the duodenum) and the tail of the pancreas were dissected out. The specimens were freeze-dried and fixed with formaldehyde vapour. Paraffin sections were subjected to an indirect immunohistochemical method for demonstrating gastrin immunoreactivity⁸. Gastrin antisera numbers 2604, 2609, 2720 and 4562 (ref. 8) were used at dilutions 1:20, 1:40, 1:80 and 1:640, respectively. As a specificity control, these antisera were inactivated by the addition of synthetic human gastrin I (100 μ g per ml diluted serum). Thus inactivated antisera failed to produce immunohistochemical staining. Samples for radioimmunoassay were taken from the same locations in 4- and 20-d-old and adult rats and extracted for 25 min in boiling redistilled water. The extracts were stored in the deep freeze until analysis. Each extract was analysed in triplicate at six different dilutions. Dilution curves for all extracts were identical with the standard curve. Gastrin was measured with antiserum 2604-8 (ref. 8). This antiserum is specific for the bioactive, C-terminal sequence of gastrin-17 and binds components I, II and III with equimolar potency⁹. It reacts very poorly with cholecystokinin, the molar ratio between ID₅₀ of synthetic human gastrin-17 and highly purified porcine cholecystokinin being <0.006. Monoiodinated gastrin-17 was used as tracer¹⁰ and synthetic human gastrin-17 as standard.

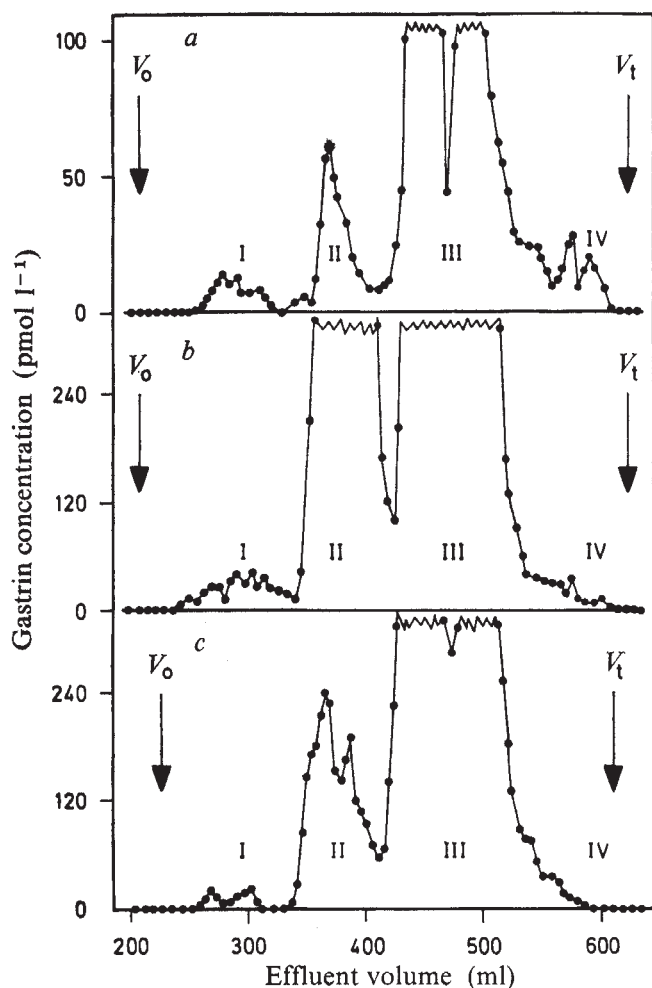


Fig. 2 Elution diagrams of gastrin components from extracts of *a*, pancreas (3.2 μ Eq synthetic human gastrin-17 per g wet weight tissue); *b*, duodenum (10.5 μ Eq synthetic human gastrin-17 per g wet weight tissue); and *c*, antrum (18.4 μ Eq synthetic human gastrin-17 per g wet weight mucosa). Pancreas and duodenum were from 4-d-old rats, antral mucosa from adult rats. The tissues were frozen and then boiled for 25 min in redistilled water, homogenised and centrifuged. The supernatants were applied to Sephadex G-50 superfine columns (25 $\times$ 2,000 mm) and eluted with 0.25 mol l^{-1} NH_4HCO_3 at 4 $^{\circ}C$ in 3.0 ml fractions at a flow rate of 15.0 ml h^{-1} . The columns were calibrated with albumin (void volume V_0), $^{22}NaCl$ (total volume V_t), highly purified human gastrin-34 (component II), gastrin-17 (component III), and gastrin-14 (component IV) donated by Professor R. A. Gregory and Dr Hilda Tracy (Liverpool). Roman numbers I–IV indicate elution volumes of components I–IV from calibration studies.

the pancreas or duodenum of adult rats. As gastrin cells are rare in the duodenum of the adult rat¹¹, however, they may therefore have escaped attention in the electron microscope.

Gastrin immunoreactivity was observed to a considerable extent in the duodenum (10.5 μ Eq synthetic human gastrin-17 per g wet weight tissue) and pancreas (3.2 μ g g^{-1}) of 4-d-old rats. The level of antral gastrin at this age was considerably lower (0.9 μ g g^{-1}). For comparison, the concentration of antral gastrin in the adult rat was 18.4 μ g g^{-1} . In the pancreas of 20-d-old rats no gastrin could be detected.

Gel-permeation chromatography revealed that gastrin immunoreactivity from the pancreas (and antrum) of 4-d-old rats eluted in the same pattern as gastrin from antral mucosa of adult rats (Fig. 2). In the duodenal extracts (4-d-old rats) the proportion of component II (gastrin-34) was greater (Fig. 2) than in the antral and pancreatic extracts.

We refer to the immunoreactive pancreatic cells as gastrin

cells because: like antral and duodenal gastrin cells they can be demonstrated in sections from formaldehyde-fixed, paraffin-embedded specimens; they can be demonstrated using a variety of gastrin antisera, of which most are directed against the bioactive C-terminal portion of gastrin-17 (ref. 9); in extracts of foetal and newborn rat pancreas significant quantities of gastrin have been demonstrated by radioimmunoassay; the extracted gastrin eluted by gel-permeation chromatography in a pattern indistinguishable from that of antral gastrin.

Little is known about the ontogeny of gastrin cells. Using a bioassay procedure Zelenkova and Gregor¹⁴ observed that in the rat antral gastrin appears first after weaning. We have reported previously¹¹ that antral gastrin as revealed by immunohistochemistry and radioimmunoassay, appears far earlier. Also, in rabbit¹⁵ and man³ gastrin cells have been shown to occur prenatally. In the 18-d-old rat foetus, gastrin cells were found in the duodenum and in the pancreas but not in the antrum. Thus, gastrin cells do not seem to appear first in antral mucosa but instead make their debut in the duodenum and in the pancreas. The role of this early gastrin is unknown, but it may exert trophic actions on the pancreas and/or the gastrointestinal tract¹⁶.

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Kinetics of reaction in calcium-activated skinned muscle fibres

A PROCEDURE reducing the diffusion-limited equilibration time of the Ca^{2+} concentration within skinned muscle fibres¹ activated from outside has been used to investigate the kinetics and the stoichiometry of Ca^{2+} in the process of tension development in frog skeletal muscle. The diffusion problems are minimised by activating (or relaxing) the myofibrillar preparation in a solution with a very high Ca^{2+} -buffering capacity compared to that of the fibre as a whole^{2,3} (solution in the interfilament space, sarcoplasmic reticulum, myofibrillar proteins); by using only thin fibres; and by using an efficient pH buffer (TES: *N*-Tris (hydroxymethyl) methyl-2-aminoethanesulphonic acid) and a

Table 1 Apparent binding constants of the ligands used in the bathing solutions

Ligand	Cation	Average apparent binding constant (M^{-1})	Experimental conditions	Observations
ATP	Mg^{2+}	$5,800 \pm 400$	Measured at pH 7.10	Very good agreement with the calculated ³ apparent binding constant based on the "absolute" values reported by Taqui and Martell ²⁸ and Phillips <i>et al.</i> ²⁹ for the same temperature values and ionic strength.
EGTA (molecular weight 380.4)	Ca^{2+}	$(5 \pm 0.5) \times 10^6$	Measured at pH 5.7–5.8, presence of TES 0–250 mM, extrapolation ³⁰ to pH 7.10	The measured apparent binding constant at room temperature was $\sim 20\%$ higher and is in good agreement with the calculated ³⁰ value based on the results of Schwarzenbach ⁷ obtained at room temperature and in the presence of 0.1 M K^+ .
	Mg^{2+}	25 ± 5	Measured at pH 7.10	The measured value at room temperature was $46 \pm 6 M^{-1}$ and is in very good agreement with the calculated value based on the result of Schwarzenbach ⁷ (see above).
HDTA (molecular weight 348.4) TES	Mg^{2+}	8 ± 1.5	Measured at pH 7.10	Agreement with Anderegg ¹⁸ .
	Ca^{2+}	6.5 ± 1		
	Mg^{2+}	≈ 1	Measured at pH 7.10	Agreement with Good <i>et al.</i> ³¹ .
	Ca^{2+}	≈ 1		
Creatine phosphate (CP)	Mg^{2+}	12 ± 1.5	Measured at pH 5.0–5.10, extrapolated to pH 7.10	Considering that at pH 7.10 the creatine phosphate is divalently charged, the apparent binding constant is in agreement with the value reported by Smith and Alberty ³² for room temperature and 0.1 M ionic strength.

The determinations were made with a pH metric method at $[K^+] = 130$ mM, $[Na^+] = 40$ mM, temperature $1 \pm 0.5^\circ C$.

high ATP concentration in conjunction with a powerful ATP regenerative system (creatine phosphate (CP) and creatine kinase (CK)). Great care has been taken in preparing bathing solutions with similar concentrations of monovalent metallic cations (~ 150 mM), protons (pH 7.1)⁴ and $[Mg^{2+}]$ (~ 1 mM) (refs 5 and 6) to those *in vivo*. For this purpose, the apparent binding constants of the various ligands for Ca^{2+} and Mg^{2+} have been measured by a pH metric method (Table 1).

A first important observation is shown in Fig. 2g, where a myofibrillar preparation was activated in a solution with a high Ca^{2+} -buffering capacity around pCa 3 (using NTA:nitrilotriacetate)^{7,8} after being equilibrated in 0.1 mM EGTA (ethanedioxybis(ethylamine)tetraacetate). Although the fibre is activated from outside⁹ force develops rapidly, with a time constant similar to that observed *in vivo* during a tetanus^{10,11}. This record can only be used for qualitative interpretations since here diffusion still remains a problem.

In the other experiments (Figs 1 and 2a–f), the Ca^{2+} -buffering capacity of the interfibrillar space has been lowered by incubating the myofibrillar preparation in 0.15 mM EGTA and by adding 10 mM caffeine to all bathing solutions¹². Subsequently, the preparations were activated in 50 mM EGTA + CaEGTA. To maintain other ionic species constant during the tension response, HDTA (hexamethylenediamine-*NNN'*-tetraacetate)¹³ was used. The loading of the skinned fibre preparation with the Ca^{2+} -sensitive photoprotein aequorin¹⁴ has indicated that for the conditions in Fig. 2a–f, the myofibrillar Ca^{2+} concentration will practically equilibrate within 150 ms of the change of solutions (unpublished).

The results presented in Figs 1 and 2a–f were obtained from the same preparation in order to reduce their scatter. These results are typical of more than 20 experiments. For the kinetic analysis it is important to note that for the experimental conditions described in the legends, the absolute steady-state level for a given $[Ca^{2+}]$ has a much more pronounced tendency to change^{15–17} (dropping by 3–8% from one contraction to the next) than the time course of the tension development, which remains essentially the same even after 7 activation–relaxation cycles (see Fig. 2 and legend). The relative steady-state isometric tension levels, unlike the absolute force values associated with different $[Ca^{2+}]$ are not affected by repetitive contractures and this relationship is illustrated in Fig. 1. A fourfold increase in

$[Ca^{2+}]$ (from pCa 6.5 to 5.88) corresponds to an increase of the relative tension from about 9 to 92%. Since the minimum number of calcium ions involved in the process of tension activation is suggested by the smallest integer n which satisfies the

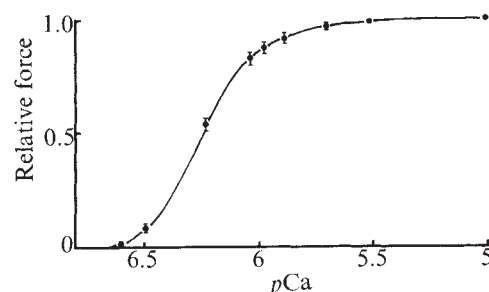


Fig. 1 Dependence of the relative steady-state isometric tension response on $[Ca^{2+}]$ expressed as pCa ($-\log[Ca^{2+}]$) in skinned fibres from the iliofibularis muscle of *Rana esculenta*. The experimental values (●) have been obtained on the same preparation, from 25 cycles of contraction (some of which are shown in Fig. 2), to reduce the scatter indicated by the vertical bars. The relative force values have been corrected for the drop in tension due to multiple activation–relaxation cycles (see Fig. 2 and text). The continuous solid line represents the theoretical prediction from equations (1)–(3) for the constants mentioned in the text. The isometric tension has been measured with an RCA force transducer. The recording system had a sensitivity of 0.3 mV μN^{-1} , a resonant frequency of 150 Hz and a compliance of 1.4 cm N^{-1} . The bathing solutions (volume 3 ml) have been changed using a method similar to that of Hellam and Podolsky^{2,3,16}. All but the incubating solution were covered to avoid volume changes. The solutions contained substances of reagent grade (mM): $[Ca-EGTA^{2-}] + [EGTA^{2-}]$, 50; $[Na_2-CP]$, 10; $[Na_2-ATP]$, 8; $[Caffeine]$, 10; $[TES]$, 140. Creatine kinase 10 U ml^{-1} . The pH was adjusted to 7.10 ± 0.01 with KOH at $1^\circ C$ so that the final value for $[K^+]$ was 137.5 ± 2 mM. The concentration of the added $MgCl_2$ varied slightly (within 1 mM) in order to maintain a free Mg concentration of 1 mM in each solution, based on the apparent affinity constants shown in Table 1. To improve the accuracy of the relative and absolute $[Ca^{2+}]$ determinations in the bathing solutions, the free EGTA concentration has been always retitrated in each solution after the experiments²⁷, and care has been taken to maintain the pH in all solutions within 0.01 units. Temperature: $2 \pm 0.5^\circ C$.

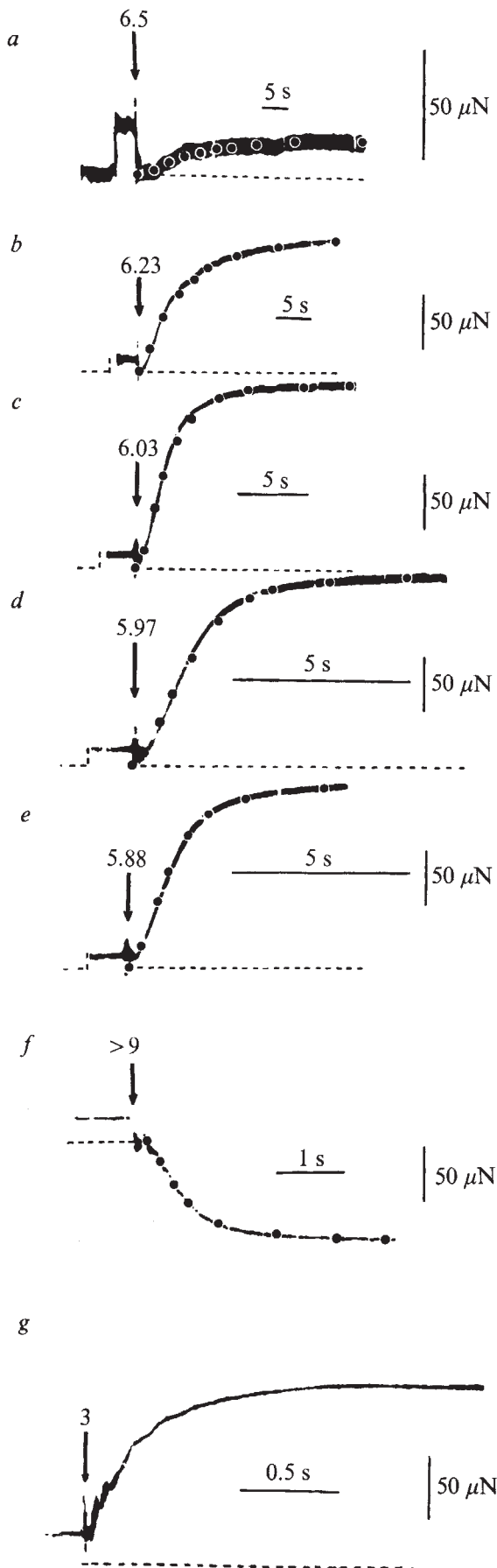


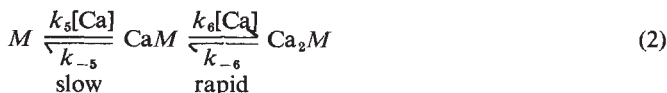
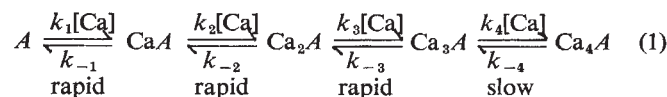
Fig. 2 *a-e* Time course of the 2nd, 4th, 5th, 6th and 7th isometric tension responses in the same frog myofibrillar bundle as for Fig. 1, when activated in EGTA containing solutions (for composition see legend Fig. 1). Diameter 50 μm , length 1.2 mm; sarcomere length close to its value *in situ*, 2.3–2.5 μm . The $p\text{Ca}$ values of the activating solutions are shown above the arrows which indicate the moment of immersing the preparation in the contracting solution. Before each contraction, the preparation has been equilibrated for at least 4 min in a relaxing solution which had the same free cationic composition as the activating solution with the exception of $[\text{Ca}^{2+}]$. The relaxing solution contained 50 mM HDTA $^{2-}$ and 0.15 mM free EGTA $^{2-}$ in order to balance the activating solutions which contained 50 mM Ca-EGTA $^{2-}$ + EGTA $^{2-}$. The contamination level of calcium was about 10 μM , due mainly to TES, caffeine and HDTA, so that the $p\text{Ca}$ value in the myofibrillar preparation before the activating step was around 8. The artefact which appears in front of the arrows is due to the movement of the myofibrillar bundle through the solution–air–solution interface. The temperature of the solutions and of the air around the preparation has been maintained to $2 \pm 0.5^\circ\text{C}$ throughout the experiment. During the first several high contraction cycles, the maximum tension had a tendency to drop by about 6% per cycle. After the 12th cycle, the steady-state values dropped less. The rate of tension development for a given $p\text{Ca}$ was more stable, decreasing for example only by 8% between the 5th and the 12th cycle of contraction, but maintaining very accurately the same shape. *f*, Time course of the relaxation (20th cycle) of the preparation in a solution containing 50 mM free EGTA $^{2-}$ ($p\text{Ca} > 9$) after being contracted for 3 min in a similar solution as the relaxing one (see above), but in which EGTA $^{2-}$ was replaced by HDTA $^{2-}$ (see text). This contracting solution contained 10 μM unbuffered Ca in addition to the contamination level of about 10 μM . *g*, Time course of the tension development in a frog myofibrillar bundle (diameter 45 μm , length 1.5 mm, sarcomere length about 2.3–2.5 μm) at 4°C , when activated in a solution containing (mM): NTA, 30; TES, 100 ($\text{pH } 7.10 \pm 0.01$ with KOH); CaCl_2 , 27.15; MgCl_2 , 6.3; K^+ , about 115; $\text{Na}_2\text{-ATP}$, 6. In this solution the level for both $[\text{Ca}^{2+}]$ and $[\text{Mg}^{2+}]$ should be close to 1 mM (refs 7, 8). The preparation had been previously equilibrated for 5 minutes in a relaxing solution containing (mM): TES, 100 ($\text{pH } 7.10 \pm 0.01$ with KOH); KCl, 86; $\text{Na}_2\text{-ATP}$, 6; MgCl_2 , 6.12; EGTA, 0.1, in which the free Mg concentration should also be about 1 mM. The dashed lines represent the level of the isometric tension in the solution before the activating (*a-e* and *g*) or the relaxing (*f*) step. The solid dots (●) superposed on the experimental records *a-f* are the predicted values for the relative tension development from the kinetic scheme presented in the text.

inequality $R'' \geq 100$ (R being the ratio between the free calcium concentration corresponding to the relative tension of 91% and that corresponding to 9% relative tension)¹⁸, it follows that in order to explain the experimental results plotted in Fig. 1, where $n = 4$, one has to assume at least 4 Ca^{2+} per 'functional unit'.

Figure 2*a-e* is a set of experimental tension records obtained as a result of a rapid increase of $[\text{Ca}^{2+}]$ in the myofibrillar bundle by the procedure described. First, up to $[\text{Ca}^{2+}] \approx 1.2 \mu\text{M}$ tension develops much slower than the expected time for $[\text{Ca}^{2+}]$ equilibration within the preparation ($< 150 \text{ ms}$, see above) and this provides a good resolution for the kinetic analysis. The fact that the relatively slow development of tension here is not due to a simple physical process such as diffusion is supported by the large variation of the apparent rate of relative tension development for different experimental conditions. Second, all the tension transients in Fig. 2*a-e* are S-shaped, that is, the maximum rate of tension development is reached a significant time after the moment of activation. This observation suggests that at least two relatively slowly equilibrating steps are involved in the chain of reactions leading to the development of tension^{3,19,20}; otherwise the curves would rise exponentially in time. A third important observation to be made is that the rate of tension development as indicated by the inverse value of the time required to reach 50% of the steady-state level, rises faster than the increase in the free calcium concentration over the $p\text{Ca}$ range shown in Fig. 2*a-e*. For an increase of 4 times in Ca^{2+} (from $p\text{Ca } 6.5$ to 5.88) the half time for the tension development decreases sevenfold. This alone suggests that at least one of the slowly equilibrating steps of reaction must involve at least 2

Ca^{2+} apparently, while the other involves at least 1. This third observation taken together with the fact that the relative tension increases over the same $p\text{Ca}$ range 6.5–5.88 such as to require at least 4 Ca^{2+} (see above) suggests that more than 4 Ca^{2+} must be involved per functional unit to produce tension. Finally, the relaxation in a 50 mM free EGTA solution ($p\text{Ca} > 9$) of the same myofibrillar preparation, which has been previously equilibrated in a solution containing about 20 μM unbuffered Ca (Fig. 2f), can be approximated with an exponential having a rate constant of -1.75 s^{-1} , a value similar to that obtained by Huxley and Simmons¹¹ *in vivo*. The time course of the relaxation is considerably faster than the time course of the activation at $p\text{Ca}$ 6.5–5.88, and this implies that an additional Ca^{2+} must be involved in a faster step of reaction, following the rate-limiting steps for activation. In addition, Fig. 2g suggests that, taken separately, the reaction steps which follow those in which Ca^{2+} is directly involved for the activation of tension should be considerably faster than the relatively slowly equilibrating steps strongly controlled by Ca^{2+} at $p\text{Ca} > 5.5$.

A detailed quantitative analysis of the results such as those presented in Figs 1 and 2, based on the assumption of reversible calcium reactions and on the suggestion that the isometric tension response in these fibres is directly correlated with the interaction between the myofilaments^{10,11}, has ruled out reaction schemes involving less than 6 Ca^{2+} per functional unit. The simplest scheme of reaction to explain the results for $p\text{Ca} > 5.5$, which can also be connected with recent biochemical data is one in which the isometric tension response, P , is proportional to the concentration of a product $\text{Ca}_4\text{A-MCa}_2$, resulting from two independent reaction schemes, each one involving one slowly equilibrating step of reaction



$$\text{and} \quad P \propto [\text{Ca}_4\text{A-MCa}_2] \propto [\text{Ca}_4\text{A}][\text{Ca}_2\text{M}] \quad (3)$$

where A-M is the tension functional unit containing the two types of Ca^{2+} reacting sites. The value of k_{-6} has been chosen such as to correspond to the rate constant of the relaxation, that is 1.75 s^{-1} , and the value for k_{-4} , suggested by the slowest rate of rise for tension (Fig. 2a), was chosen as 0.06 s^{-1} . The values for the other parameters used for explaining the experimental results in Figs 1 and 2 are: $k_{-1}, k_{-2}, k_{-3} \gg 15 \text{ s}^{-1}$; $100K_1 < K_2$; $10^4 K_3 < K_4$; $K_1 K_2 = 7 \times 10^{11} \text{ M}^{-2}$; $K_3 K_4 = 2.02 \times 10^{13} \text{ M}^{-2}$ ($K_i = k_i/k_{-i}$ for $i = 1, 2, 3, 4$); $k_5 = 9.44 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$; $k_{-5} = 8.75 \text{ s}^{-1}$; $k_6 = 3.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$. The predictions of this kinetic scheme have been superposed over the experimental results in Figs 1 and 2a-f. One can see that the agreement between theory and experiment is good.

Recent experiments suggest that the skeletal muscle in vertebrates might have a Ca^{2+} control mechanism of contraction linked with both myosin and actin filaments²¹⁻²⁴. There is also evidence that the actin control could involve 4 Ca^{2+} per troponin^{25,26}. The kinetic scheme presented here based solely on experiments using skinned frog muscle fibres in which $[\text{Ca}^{2+}]$ has been rapidly changed can be attractively connected with this new biochemical evidence, and suggests in addition that the myosin control in frog might involve two Ca^{2+} per functional unit.

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X-ray titration of binding of β, γ -imido-ATP to myosin in insect flight muscle

We have attempted to establish a correlation between the binding of an ATP analogue, β, γ -imido-ATP (hereafter termed imido-ATP), and a structural change induced in muscle in the presence of this analogue. Imido-ATP binds tightly at the enzymatic site of myosin, but is not hydrolysed there¹. When applied to glycerol-extracted muscle, it binds to the myosin *in situ* without causing the muscle to relax—that is, a ternary actin–myosin–nucleotide complex is formed². Muscle fibres in this condition have the same mechanical stiffness as in rigor, but their zero tension point is displaced to a slightly greater muscle length, and both X-ray diffraction and electron microscopy indicate that the conformation of the attached myosin has altered, as if the head was rotated relative to the actin^{2–5}.

Unfortunately both structural signs can be faulted, the electron microscopy because of the possibility of fixation or postfixation changes⁶, and the X-ray diffraction because of the long time which was used to obtain the required exposures^{2,4}. We have tried to overcome these objections by observing diffraction changes within a short time after the nucleotide was added, and checking their relevance to the nucleotide binding by their relationship to the concentration used—that is, to obtain an X-ray titration of the nucleotide binding. For this purpose, the most powerful source of X rays available was used. The experiments were carried out at the EMBL synchrotron radiation facility at the DESY synchrotron, Hamburg^{7,8}, on glycerol-extracted dorsal longitudinal flight muscle from *Lethocerus cordofanus* dissected into bundles of 25 to 40 fibres. These were mounted on a mechanical apparatus in which their stiffness and tension could be monitored⁹, and immersed in a

neutral, calcium-free buffer at low ionic strength at 13–15 °C (20 mM imidazole, 10 mM NaN_3 , 4 mM EGTA, 10 mM MgCl_2 , pH 6.9, as described previously⁴). An intense monochromatic X-ray beam of wavelength 1.5 Å was passed through the sample and the resultant small angle diffraction pattern from the hexagonal lattice of myosin and actin filaments was recorded at 80-cm specimen-counter distance using a position-sensitive gas detector with a resolution of approximately 0.2 mm full width at half maximum (FWHM) (ref. 9).

The two strongest diffraction peaks on the equator deriving from the basic 530 Å lattice periodicity and designated 10 $\bar{1}$ 0 and 20 $\bar{2}$ 0 were readily recorded in conveniently short times. By slight displacement of the beamstop, it was possible to observe one 10 $\bar{1}$ 0 and both 20 $\bar{2}$ 0 diffraction peaks clearly (Fig. 1a). The entrance slit of the detector was opened wide (2 mm) so that the entire width of the equatorial peaks were received; the efficacy of this was checked by comparison of the counting scans with densitometer traces from films of the same specimens. Data were usually collected in 300 s, representing $0.5\text{--}1.0 \times 10^4$ counts per channel at the peak of each reflection. The intensity of each of the three peaks was estimated from the product of its peak height above background and half-height width. The 20 $\bar{2}$ 0/10 $\bar{1}$ 0 intensity ratio R was calculated from the mean value of the two outer peaks, $R = (20\bar{2}0_L + 20\bar{2}0_R)/2 \times (10\bar{1}0)_R$.

In rigor (that is, in the absence of nucleotide) R was slightly greater than unity (mean $\pm$ s.e. 1.26 ± 0.03 ; $n = 27$), which agrees with previous estimates¹⁰. On addition of sufficient imido-ATP to ensure binding to myosin², the ratio fell by approximately one-third of its value (Table 1). The widths of the peaks did not alter significantly. Two-thirds of the change in R occurred before the mid-time of the first measurement (3–6 min after addition of the nucleotide), the remaining change occurring gradually over the ensuing 20 min. On removal of the nucleotide, the change was partially reversed (mean recovery $60 \pm 10\%$; $n = 10$); again, approximately two-thirds of the reversal occurred 5–7 min after the start of removal and the rest over the following 30 min. Sequential increases of nucleotide concentration at 10-min intervals produced a graded increase in R . The data fitted a hyperbolic binding curve (Fig. 1b) with a dissociation constant (Table 1) similar to that obtained by direct binding measurements². On removal of the nucleotide after the 2-h immersion necessary to obtain this curve, the ratio

Fig. 1 *a*, Trace of output from position-sensitive detector aligned along the equator of the diffraction pattern from insect flight muscle in rigor. The 10 $\bar{1}$ 0 peak on the left-hand side of the diagram is elevated by the background and interrupted by the beamstop; it was not used for calculation. *b*, Change in R on addition of β, γ -imido-ATP in a single experiment. The continuous line is the best fitting hyperbola ($K_d = 65 \pm 6 \mu\text{M}$, $\Delta R_{\text{max}} = 0.31 \pm 0.01$) (ref. 18).

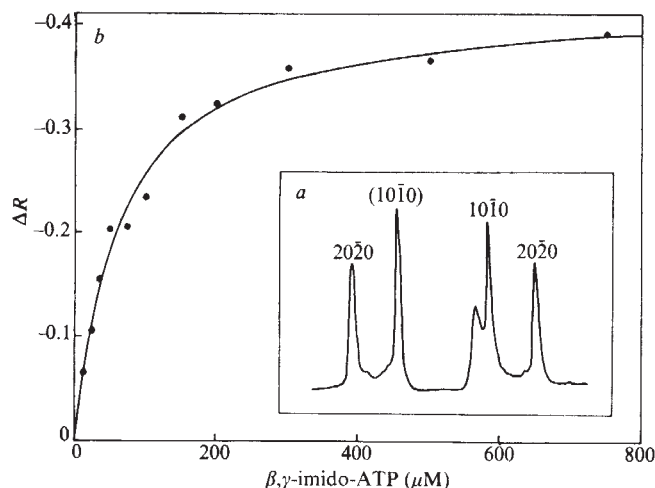


Table 1 Changes in R on adding various ligands to insect flight muscle

Ligand	ΔR
β, γ -imido-ATP	
500–1,700 μM	-0.34 ± 0.05 (10)
Limiting value*	-0.41 ± 0.03 (46); $K_d = 90 \pm 19 \mu\text{M}$
ADP 200–400 μM †	-0.01 ± 0.01 (7)
Pyrophosphate	
600–1,200 μM	-0.17 ± 0.03 (4)
Limiting value†	-0.25 ± 0.04 (16); $K_d = 450 \pm 120 \mu\text{M}$
ATP 15 mM	-0.59 ± 0.07 (5)

Mean $\pm$ s.e. cited; number of experiments in brackets. ΔR was calculated from the change in peak heights of the two reflections on adding the ligand.

* Calculated by hyperbolic fitting to the measurements at different ligand concentrations¹⁸. 10 mM Mg^{2+} present in all solutions except ATP, where 15 mM Mg^{2+} was added; pH 6.9, ionic strength = 0.1, 14 °C.

† 50–100 μM diadenosyl pentaphosphate was also added to these solutions to block adenylate kinase activity²⁰.

reverted towards the rigor value, as in the shorter immersions.

The effect of other ligands was also studied. ADP, in a concentration well above its known dissociation constant¹¹, had no significant effect on R (Table 1). Pyrophosphate reduced R by a smaller amount than imido-ATP at comparable concentrations. Its effect was graded over the range available, which was limited by the solubility of Mg -pyrophosphate. Application of a hyperbolic fit indicated that the full effect of binding pyrophosphate was probably greater than that observed, but still less than that for imido-ATP (Table 1). On removal of pyrophosphate the ratio reverted to or beyond its initial value (mean recovery $125 \pm 25\%$; $n = 4$).

In all these cases the 5-Hz stiffness (that is, muscle stiffness measured by the change in tension resulting from a small amplitude 5-Hz sinusoidal length oscillation) of the muscle remained high (approximately 20 mg per fibre % extension) in the presence of nucleotide, as expected from previous results². On addition of ATP the muscle relaxed; the 5-Hz stiffness fell by a factor of four and could not be restored by extension. The ratio R fell by approximately half its value (Table 1) in agreement with previous observations¹⁰; the widths of the peaks did not alter. The effect was reversed by removal of the nucleotide (mean recovery $87 \pm 3\%$; $n = 3$).

During the course of each of these experiments, which lasted 3–15 h, the filament lattice spacing gradually decreased by approximately 5% (mean initial value $533 \pm 5 \text{ Å}$; $n = 5$; final value $510 \pm 4 \text{ Å}$; $n = 5$). Addition of any of the unhydrolysed ligands had little or no effect on the spacing.

These results show that within a short time of the binding of imido-ATP to myosin, a structural change occurs in the muscle, the magnitude of which is directly proportional to the amount of nucleotide bound. They do not show the nature of the change, since according to model studies either rotation of the attached myosin, redistribution of the locus of attachment along the actin filament or detachment of a proportion of the myosin from the actin would all cause such a change (refs 10 and 12, and K. C. Holmes, J. B. L. and R. T. T., unpublished). We believe that the third possibility is excluded by the retention of the muscle's stiffness and the high intensities of the actin-based layer lines in the presence of imido-ATP^{2–4}. It is not possible to differentiate the other two possibilities. Whichever structural change is responsible, it certainly occurs quickly; the main objection to the X-ray diffraction evidence has been removed.

It is probable that the change in R produced by pyrophosphate has the same molecular origin, since electron microscopy¹³ shows elements of the same changes occurring in the muscle. The observed concentration dependence agrees with that deduced from mechanical observations¹⁴. The failure of ADP to shift the ratio indicates that this nucleotide causes relatively little redistribution or rotation of the myosin; the outer layer lines of

the X-ray diffraction pattern are also little altered by this ligand¹⁵.

These results are also relevant to vertebrate skeletal muscle, since Lynn has shown that imido-ATP produces a large shift in the 1120/1010 ratio in glycerol-extracted rabbit psoas fibres¹⁶. As imido-ATP does not reduce the 5-Hz stiffness of these fibres at a given tension², it is probable that this change is also due to a redistribution or rotation of attached myosin, rather than its release from the actin. If so, this invalidates the assumption that a change in *R* can be used to predict the proportion of myosin attached to actin^{17,19}.

In conclusion, we now have evidence that ligands which attach to the enzymatic site of myosin do alter either its position relative to the actin or the location of the actin to which it tends to attach. The effect is both rapid and specific, and may well be relevant to the state of the myosin during its contractile cycle.

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Phosphorylation of troponin I and the inotropic effect of adrenaline in the perfused rabbit heart

THE regulation of contractile activity in cardiac muscle by changes in the intracellular Ca^{2+} concentration involves the regulatory protein system, consisting of tropomyosin and the troponin complex that is located in the I filament. Although the regulatory protein system of cardiac muscle is essentially similar in function to that of skeletal muscle, the components differ in the two tissues. Cardiac tropomyosin consists principally of α subunits, whereas the skeletal protein is composed of α and β subunits¹. Troponin I, troponin C and troponin T are all specific for cardiac muscle in that they have different chemical and immunological properties from the corresponding skeletal muscle proteins^{2–4}. In addition, cardiac troponin I and cardiac

troponin C have been shown to have different amino acid sequences from their skeletal muscle counterparts^{3,5}.

Although rabbit cardiac troponin I shows considerable sequence homology with troponin I from fast skeletal muscle, it has an additional 26 residues at the N terminus³. The rapid phosphorylation of a serine at position 20 in this extra N-terminal part of the cardiac troponin I molecule is mainly responsible for the fact that the phosphorylation catalysed by cardiac cyclic AMP-dependent protein kinase is about 30 times faster with cardiac troponin I than with fast skeletal troponin I (ref. 4). When isolated immediately after death by stunning, a phosphate content of 2–3 mol mol⁻¹ has been reported for cardiac troponin I, compared with 0.5–1.0 mol mol⁻¹ for skeletal muscle troponin I (ref. 4). These differences suggest that the phosphorylation of troponin I may have a special significance for the regulation of the contractile activity of cardiac muscle. Further evidence for such a role indicates that there is a correlation between the contractile force developed on adrenaline application and the extent of phosphorylation of troponin I in the perfused rat heart⁶.

We describe here investigations extending our studies on the enzymic phosphorylation of isolated rabbit cardiac troponin I to the perfused heart. We have attempted to relate the effects of adrenaline on the contractile activity of the perfused rabbit heart to the phosphorylation of specific sites on the troponin I.

The total phosphate content of rabbit cardiac troponin I varied with the conditions in which the heart was removed. Troponin I was immediately isolated by affinity chromatography⁷ and, before analysis, was washed four times with 7.5% (w/v) cold trichloroacetic acid to remove non-covalently bound phosphate. Values of 2.1 ± 0.4 mol P per mol were obtained when hearts were removed immediately after death by stunning. If hearts were made anoxic by clamping the aorta for 30 s just before removal from the animal, similar or slightly higher values were obtained. If the rabbits were anaesthetised with pentobarbital, and troponin I subsequently isolated from hearts removed after freezing *in situ* in Wollenberger clamps chilled with liquid nitrogen, a value of 1.5 ± 0.2 mol P per mol troponin I was obtained.

Hearts removed rapidly from stunned rabbits, as described⁸ for rats, were cooled for 15–20 s in ice-cold saline and perfused by the Langendorff procedure at 37 °C with modified Krebs–Henseleit buffer⁹ in an apparatus fitted with a recirculation chamber similar to that described by Morgan *et al.*¹⁰. Force and rate were recorded on a polygraph using a force transducer attached to a stainless steel hook in the apex of the heart. At various times, the ventricles (about 4 g tissue) were cut rapidly from the heart and homogenised immediately in 30 ml of 9 M urea, 75 mM Tris-HCl, pH 8.0, 1 mM CaCl_2 , 15 mM β -mercaptoethanol and the troponin I isolated by affinity chromatography⁷. During the 2–5 min initial equilibration period, cardiac troponin I contained 2.5 ± 0.4 mol P per mol. After 10–15 min perfusion, the phosphate content of troponin I fell to 1.5–1.6 mol P per mol and remained nearly constant for at least 100 min, during which heart rate and force also remained virtually unchanged.

Addition of 0.1–4.0 μM adrenaline to the perfusion media after an initial equilibration period of 15 min caused a rapid and dose-dependent increase in the force developed by the myocardium. Hearts were removed rapidly from the apparatus at the moment of peak force development, homogenised and troponin I isolated. Total phosphate content increased in parallel with increasing force, reaching a maximum of about 3 mol P per mol troponin I at 2 μM adrenaline, when the maximum contractile response was obtained (Fig. 1).

If troponin I was isolated 5–10 min after the maximum inotropic response when the contractile force had returned

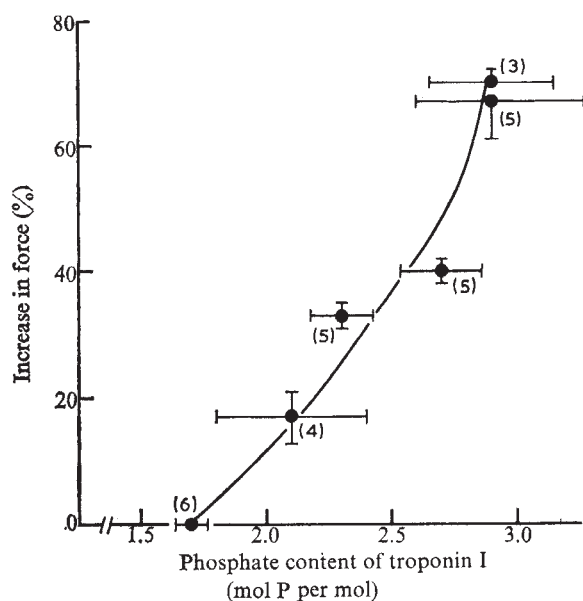


Fig. 1 Relationship between the phosphate content of troponin I and the increase in force developed in the perfused rabbit heart. Force changes were induced by adding $0.3\text{--}4.0\ \mu\text{M}$ adrenaline to modified Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 0.2 mM KH_2PO_4 , 0.5 mM Na_2EDTA , 25 mM NaHCO_3 and 5 mM glucose) equilibrated with 95% O_2 :5% CO_2 at 37°C , pH 7.4. Hearts were first perfused for a 15-min equilibration period with normal buffer and then switched to buffer containing adrenaline. At the peak of the force change, the hearts were homogenised rapidly in buffered 9 M urea and troponin I isolated as described in the text. Phosphate content was calculated from total phosphate and nitrogen contents measured in duplicate samples of trichloroacetic acid-washed troponin I as described previously¹⁵, using a molecular weight of 22,500 and a nitrogen content of 16% for cardiac troponin I. Figures in parentheses indicate number of hearts analysed.

to a value similar to that before adrenaline application, the total P content was similar to that obtained after equilibration but before addition of adrenaline. In the presence of β -adrenergic blockers such as propranolol, the inotropic response to adrenaline was inhibited and no significant rise in the phosphate content of troponin I occurred.

In a parallel series of experiments, the troponin I isolated after perfusion was washed repeatedly with cold 7.5% trichloroacetic acid to remove non-covalently bound phosphate and digested with cyanogen bromide. In control hearts perfused for 20 min, 90–95% of the total covalently bound phosphate was present in the N-terminal cyanogen bromide peptide of cardiac troponin I that contains residues 1–48 (ref. 3).

After 15–20 min perfusion with Krebs-Henseleit-bicarbonate medium containing phosphate labelled with ^{32}P , the N-terminal cyanogen bromide peptide became radioactively labelled. Of the ^{32}P recovered from a tryptic digest of this N-terminal peptide 80–90% was bound to serine 20, indicating that in the perfused beating heart there was rapid exchange between the pool of intracellular phosphate compounds and the phosphate on serine 20. If the troponin I was isolated at the peak of the inotropic response to adrenaline, the total incorporation of ^{32}P increased. The distribution of radioisotope in the peptides present in the cyanogen bromide digest of this troponin I indicated that 90–95% of the total ^{32}P incorporated was associated with the region of the cardiac troponin I molecule represented by residues 1–48.

These results suggest that at least three phosphorylation sites are significant for the function of cardiac troponin I in the rabbit. One of these, serine 20, is readily phosphorylated by cyclic AMP-dependent protein kinase. This site is

usually phosphorylated in the perfused heart and readily exchanges with the intracellular phosphate pool. The precise location in the region consisting of residues 1–48, of the other two sites that must be phosphorylated when the total phosphate content rises to 3 mol mol^{-1} , is not clear. The sequence studies of Grand *et al.*³, have shown that there are serines at positions 4, 20, 32, 37 and 39 and threonines at positions 26 and 46. Of these, serine 37 might be expected to be a site for phosphorylation by phosphorylase kinase by analogy with the action of this enzyme on troponin I from fast skeletal muscle of the rabbit^{11,12}. As yet we have no information as to whether phosphorylation of serine 37 is involved in the inotropic response.

The results indicate, however, that the increase in the phosphate content of troponin I from about 2 mol mol^{-1} to 3 mol mol^{-1} is closely associated with the increase in contractile force obtained in cardiac muscle treated with adrenaline.

In troponin I from fast skeletal muscle, the N-terminal region is somehow involved with interaction with troponin C (ref. 13). Judging from the sequence data of Grand *et al.*³, and the inhibition of the phosphorylase kinase-catalysed phosphorylation of cardiac troponin I by troponin C (ref. 4), a similar interaction probably occurs in the cardiac system. The introduction of negative charges by phosphorylation in the N-terminal region of the molecule, which contains an excess of positively charged amino acids, would be expected to alter the strength of the interaction with the negatively charged troponin C and thereby possibly change the affinity of troponin C for Ca^{2+} .

Preliminary investigations in this laboratory on natural and desensitised actomyosin indicate that after phosphorylation of cardiac troponin I by cyclic AMP-dependent protein kinase, the Ca^{2+} concentration required for 50% activation of the ATPase is increased, that is, the system is less sensitive to Ca^{2+} concentration (H. A. Cole and S.V.P., unpublished). This finding does not confirm those of Rubio *et al.*¹⁴, who reported an increase in Ca^{2+} sensitivity of the ATPase of cardiac natural actomyosin on phosphorylation of troponin I.

Although our results do not permit us to say whether there is a direct casual relationship between the phosphorylation of troponin I and the inotropic effect of adrenaline, the events are undoubtedly closely associated in time. In view of the results of the enzymic studies, we suggest that one function of phosphorylation of troponin I is to act as a negative feedback system. Thus by reducing the sensitivity of the actomyosin ATPase to Ca^{2+} , it smoothes out the contractile response to the rise in intracellular Ca^{2+} which results from the action of adrenaline on the myocardium. A detailed account of this work will be published elsewhere in due course.

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Increase in human skeletal muscle lactate produced by fenfluramine

OUR recent hypothesis¹ that "anorectic agents" such as fenfluramine and mazindol owe at least part of their anti-obesity properties to a peripheral action on glucose metabolism by causing an increase in glucose uptake into skeletal muscle and a subsequent "wasting of calories" has been criticised² on the grounds that there is no information on the fate of the glucose so taken up, nor convincing evidence for an increase in metabolic rate with these drugs. We have now studied the effect of fenfluramine on lactate production in isolated human skeletal muscle, our previous work having shown no increase in glycogen levels³ nor in carbon dioxide production⁴. An increase in lactate production would indicate metabolism of the glucose entering the cell under the influence of fenfluramine.

Human skeletal muscle was obtained at surgery for total hip joint replacement. The preparation and incubation of the tissue (in Krebs-bicarbonate buffer with added bovine serum albumin 2 mg ml⁻¹ and glucose 16.67 × 10⁻³ M) was carried out using the method of Kirby, Leighton and Turner⁵. Muscle strips, wet weight 80–140 mg, were prepared from each sample and incubated in 2 ml of media containing (1) 100 µU ml⁻¹ insulin, (2) 100 µU ml⁻¹ insulin plus 100 ng ml⁻¹ fenfluramine (previously shown to cause a maximal increase in glucose uptake⁶), (3) 100 µU ml⁻¹ insulin plus 100 ng ml⁻¹ amphetamine (amphetamine has no significant effect on glucose uptake into isolated muscle³). For each muscle sample two strips were prepared and used for control lactate determinations; they were not incubated. Flasks were flushed with a mixture of 95% oxygen and 5% carbon dioxide for 10 s before incubation at 37 °C for 90 min. The lactate content of the muscle and the residual media were then determined.

The method for measurement of muscle and residual media lactate was based on that for blood lactate using NAD/LDH enzymes⁷ and the Boehringer Mannheim Biochemica Test Combination for lactate. Muscle strips

were removed from the media and homogenised for 5 min at 25,000 r.p.m. with a blade homogeniser in 2 ml of 0.6 M perchloric acid. The homogenate was centrifuged for 10 min at 3,000 r.p.m. and 0.2 ml of the supernatant used for the lactate determination. One millilitre of the residual medium was added to 1 ml of 0.6 M perchloric acid and centrifuged as before, 0.2 ml of the supernatant being used for the subsequent analysis, with the Biochemica Test Combination. A lactate standard (0.5 mM) was included as a blank. Preliminary work had demonstrated a linear relationship between absorbance at 340 nm and lactate concentration over the range 0–1.0 mM. Good reproducibility was obtained with the method, the mean of six muscle lactate determinations in strips from the same sample being 6.91 ± 0.21 mmol per kg wet weight of muscle.

The results (Table 1) demonstrate that fenfluramine produced an increase in muscle lactate concentrations. Amphetamine, which has no effect on glucose uptake, has no significant effect on lactate concentration. Further studies are needed to determine in more detail the metabolic fate of the glucose taken up under the influence of fenfluramine, but these results are further evidence for a peripheral metabolic mechanism in its anti-obesity activity.

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Ribosome slowed by mutation to streptomycin resistance

ALTHOUGH the basic mechanisms of protein synthesis are now rather well understood, particularly in *Escherichia coli*, it is less clear how the high accuracy of this process is achieved. (The error rate in protein synthesis in *E. coli* seems to be about one in 10⁴ amino acid misincorporations (Edelman and Gallant, unpublished results, and our own unpublished data)). A major site determining accuracy in protein synthesis is the ribosome, and in addition to the ribosome-mediated effects on accuracy (*in vivo* and *in vitro*) of various metal ions, aminoglycoside antibiotics and organic solvents, there are ribosomal mutations which increase or decrease accuracy¹. Certain mutations of the 30S ribosomal protein S12 conferring streptomycin resistance (the *strA* locus) enhance accuracy^{2–5}, whereas certain mutations in the S4 protein (the *ram* locus) are known to decrease accuracy⁶. The *strA* mutations are restrictive (reducing) in their effect on both missense and nonsense suppression *in vivo* and on miscoding *in vitro*³, the *ram* mutation, on the other hand, has the opposite effect in both cases⁶. It is possible that it is the kinetics of polypeptide synthesis at the ribosome that determines accuracy and that the kinetics in turn are affected by the *strA* and *ram* mutations. Because the same transfer RNA (tRNA) discrimination kinetics that may determine accuracy may also contribute to the elongation speed, we have investigated the effect of mutation to streptomycin resistance on the speed of polypeptide elongation.

A simple kinetic model for discrimination of charged

Table 1 Lactate formed by isolated human skeletal muscle incubated with insulin, fenfluramine or amphetamine

Insulin*	Fenfluramine plus insulin: change compared with insulin alone*	Amphetamine plus insulin: change compared with insulin alone*
13.85	+1.04	–0.10
14.11	–2.09	+1.82
9.79	+2.33	+0.84
9.12	+0.23	–3.00
10.97	+0.10	–0.53
8.78	+1.67	+2.07
7.68	+1.07	–1.67
8.58	+1.53	–1.57
7.48	+2.53	–0.08
8.79	+1.07	–0.70
Means ± s.e.m.	+0.95 ± 0.42	–0.29 ± 0.50
<i>t</i>	2.26	0.58
Significance	<i>P</i> = 0.05	NS

All values are means of two incubations.

*mmol lactate per kg wet weight skeletal muscle per 90 min; 100 µU ml⁻¹ insulin, 100 ng ml⁻¹ fenfluramine and 100 ng ml⁻¹ amphetamine.

tRNA at the ribosome, like that described by Ninio⁷, may be constructed. Such models imply directly that the more accurate a given ribosome, the slower is the transpeptidation reaction, and therefore the longer is the time required to synthesise a polypeptide of given length. In such a model one can attribute the specificity of message-directed polypeptide synthesis to the energy differences between binding of the complementary and non-complementary aminoacyl-tRNAs to the message codon and the amino-acyl site on the ribosome. Although we do not know what part of the elongation cycle time is required for transpeptidation, we expect that a mutation to streptomycin resistance, and therefore to a more accurate ribosome, should cause an increase in this time and thus a slowdown in elongation. We investigated this hypothesis by examining an otherwise isogenic set of independent spontaneous mutants, resistant to high levels of streptomycin, and have found that they do translate message at a slower rate.

Translation speed was determined by measuring the time required for the first newly synthesised β -galactosidase molecules to appear after induction of the lactose operon. This induction lag was measured in exponentially growing cultures by sampling every 5 (or 10) s into chloramphenicol to stop protein synthesis and assaying each of these samples for β -gal activity to generate an induction curve. The time between induction and the appearance of the first newly synthesised enzyme was determined to an accuracy of ± 3 s. For the wild type, streptomycin-sensitive strain (IH79), the lag time we measured agrees with the results of Lacroute and Stent⁹ and Kepes¹⁰. Although we are interested here in the relative differences in the measured lag times and not necessarily the absolute chain elongation times, the results of their experiments^{9,10} indicate that the chain elongation

Fig. 1 Measurement of lag time to first completed β -galactosidase molecule (β -gal). Cultures of a lac permease negative (derivative of W1485) K12 strain, IH79(*Str*^S), and a spontaneous mutant derivative to streptomycin (200 μ g ml⁻¹) resistance, IH82, were grown in glycerol minimal medium at 37 °C to a well-established exponential phase and then induced at $t=0$ by adding isopropyl- β -D-thiogalactopyranoside to a concentration of 1.0 mM. The cultures were sampled every 5 s by taking 0.5 ml into test tubes containing 0.5 ml of a solution (1.0 mg ml⁻¹) of chloramphenicol in distilled water. These samples were then assayed for β -galactosidase activity. Cell lysis was accomplished with chloroform and β -gal activity measured by hydrolysis of orthonitrophenol- β -D-galactopyranoside at 37 °C (ref. 8). The data are reported here in terms of the uninduced level of β -gal. From the observed point to point variation in the measured basal level (points before 80 s, for example) and by repeating the experiment several times we determined the inherent error in specifying the intercept and estimate that the time lag was measured to an accuracy of ± 3 s. Δ , IH32, streptomycin resistant; $\circ$, IH79, streptomycin-sensitive.

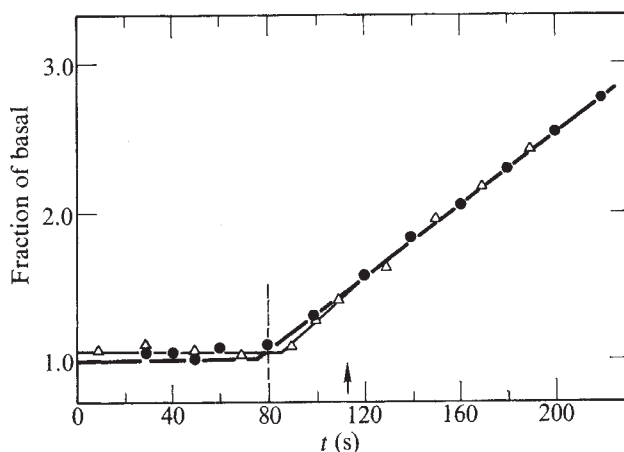
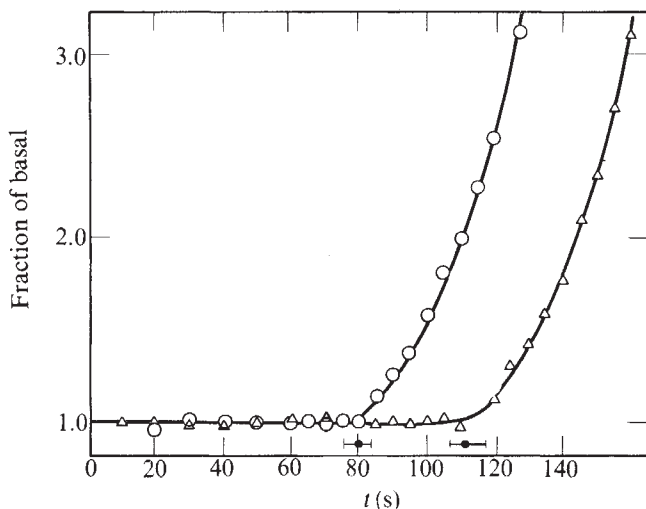


Fig. 2 Induction curves for IH79 and IH82 with actinomycin D. The conditions and sampling procedures for the chloramphenicol experiments were as described in the legend to Fig. 1. The cultures for the actinomycin experiments were treated by resuspension at 37 °C in EDTA-Tris buffer¹³ (1 mM EDTA, 0.12 M Tris, 1 mM Na₂PO₄, 0.5% glycerol at pH 7.6) for 2 min. The EDTA-treated culture was then diluted 10:1 into prewarmed medium. The induction was begun 5 min after dilution. The sampling scheme was as in the chloramphenicol experiments except that the samples were held at 37 °C for 30 min after sampling to permit all free message to be completely translated. Δ , Strain IH82 (*Str*^R); $\circ$, IH79 (*Str*^S). The intercepts are not significantly different from each other or the chloramphenicol measured intercepts for IH79 (Fig. 1) (into 0.5 ml of actinomycin D 20 μ g ml⁻¹). ---, Lag time for IH79; arrow, lag time for IH82 (Fig. 1).

time is at least 90% of the lag time (see also our unpublished work).

We isolated a set of streptomycin-resistant mutants (to 200 μ g ml⁻¹ of streptomycin) which were produced spontaneously from independent cultures. This strain set is therefore, with high confidence, isogenic for all loci but the *strA* locus. Identical procedures for measuring the induction lag revealed a significant increase in the lag time for the streptomycin-resistant derivatives of IH79 over the parent strain (see Fig. 1 in which data for the parent and one such derivative are presented). The independence of the *strA* mutations in this set, the negligible probability of double mutation and the well characterised nature of the *strA* locus as the structural gene for the 30S ribosomal protein S12 and the only mutational locus for the high level streptomycin resistance¹¹, strongly suggest that the change in speed of protein synthesis is caused by a change in the ribosome speed during translation, rather than by a change in transcription rate.

This conclusion can be tested further by measuring the lag time for the completion of the first messenger RNA (mRNA) transcript of the gene. We have measured this time by using the DNA-binding drug actinomycin-D to stop transcription in a set of samples taken in a time series identical to that described above¹². The time lag observed by this method (see legend to Fig. 2) is the time required for the passage of the RNA polymerase molecule down the full length of the *z* gene, the time for completion of the initial mRNA. The results (shown in Fig. 2) revealed no significant difference between sensitive and resistant strains and support our presumption that the mutation to streptomycin resistance affects the elongation speed and leaves the transcription speed essentially unchanged.

There is evidence that translation speed and transcription speed are coupled¹⁴ by some yet unknown mechanism. Judging from the results of Gupta and Schlessinger¹⁴ we would expect, however, to see a significant shift in transcription speed only for translation speeds much less than we observe in IH82.

These experiments show clearly that ribosome speed in *E. coli* is not a fixed parameter inherent to the protein

synthetic apparatus, but a variable determined by the kinetics of translation and ultimately by the structure of the ribosome. (After this work was completed another, possibly related, phenomenon was reported in which increased levels of streptomycin in streptomycin-dependent strains apparently cause a proportional increase in the speed of translation¹⁴.) We have observed a shift from about 14.7 amino acids per s in the wild type (*Str*⁺) to about 10.5 amino acids per s in some ribosomal mutants (based on a β -galactosidase length of approximately 1,173 amino acids¹⁵). Furthermore, the correlations between speed and ribosomal accuracy which are expected from simple kinetic models are in agreement with the observations that mutation to streptomycin resistance but not to spectinomycin resistance (data not shown), which does not affect ribosomal accuracy, increases accuracy and decreases ribosome speed. The nature of the delayed induction curve seen in the streptomycin-resistant strains suggests that the delay is caused by a change in elongation speed and not in the ribosomal initiation rate: a decreased initiation frequency would cause a decreased rate of β -gal synthesis, not just a delay in an otherwise unchanged induction curve as we observe. A more direct separation of the two effects will, however, be necessary before this question can be settled completely.

Further experiments, designed to investigate the accuracy-speed connection suggested by this work, have been carried out on previously characterised *strA* alleles and on a characterised *ram* allele. This work (to be reported elsewhere) indicates that the *ram* allele reverses the effect of mutation to *Str* resistance, and that *strA* alleles may be ordered by their ribosome speeds. Further biochemical and genetic investigations of the kinetics of translation may lead to a fundamental understanding of the molecular processes underlying the discriminatory functions central to the information transfer which occurs at the ribosome during protein synthesis.

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Regulation of ribosomal RNA methylation in a temperature-sensitive mutant of BHK cells

CONSERVATION of methylated sequences of mammalian ribosomal precursor RNA (pre-rRNA) into mature cytoplasmic ribosomes suggests an essential structural or regulatory function for these modified bases¹⁻³, although the role of post-transcriptional methylation is not clear. At the non-permissive temperature (39 °C), *ts 422E*, a temperature-sensitive mutant of BHK 21/13 cells⁴⁻⁶, is deficient in 28S rRNA maturation and assembly of 60S

ribosomal subunits, although transcription of 45S pre-rRNA and cytoplasmic appearance of 18S rRNA and 40S subunits remains unimpaired³. To examine the relationship between RNA methylation and the processing of ribosomes, we have studied this mutant and find it contains methyl-deficient pre-rRNA. Our results show an expanded pool of nuclear 45S pre-rRNA molecules in the mutant at 39 °C, suggesting an inhibition of its processing to 32S RNA. In addition, 45S and 32S precursor molecules in *ts 422E* are substantially undermethylated relative to the pre-rRNAs of wild-type BHK cells at 39 °C but identical at the permissive temperature (33 °C). The residual amount of mature

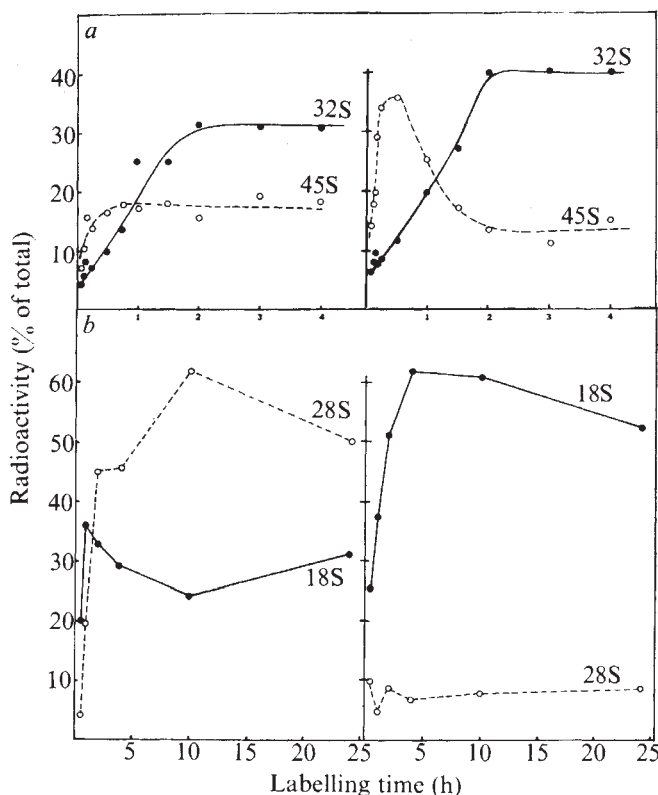


Fig. 1 Accumulation of nuclear (a) and cytoplasmic (b) ribosomal RNA in *ts 442E* and BHK cells. Monolayer cultures of wild-type BHK (left panels) and *ts 422E* cells (right panels) were shifted from 33 to 39 °C, and after 12 h were labelled with 25 μ Ci ml⁻¹ 5,6-³H-uridine (37.6 Ci mmol⁻¹) in Dulbecco-Vogt Modified Eagle's Medium (DME) supplemented with 3 μ M uridine plus 10% calf serum. Collected cells were lysed in 2% citric acid, 0.5% Triton X-100 (Rohm and Haas, Philadelphia) and nuclei were deposited and washed twice with the same buffer by centrifugation at 800g for 2 min. Nuclear pellets suspended in 10 mM Tris-HCl (pH 9.0), 0.2 M NaCl, 10 mM EDTA, 0.5% SDS (NETS buffer) were extruded through a 22 gauge needle to shear DNA then alternately extracted at room temperature with phenol:chloroform:isoamyl alcohol (50:50:1) and chloroform:isoamylalcohol (24:1) until the interfacial layer was clear of denatured protein¹⁸. Post-mitochondrial supernatants prepared from cells lysed as described previously¹⁹ by centrifugation at 10,000 r.p.m. for 10 min in the Sorvall SS-34 rotor, were adjusted to 70 mM MgCl₂ for 45 min at 0 °C to precipitate cytoplasmic ribonucleoproteins (RNP)²⁰. Ribosomes pelleted by centrifugation at 10,000 r.p.m. for 10 min were dissolved in NETS buffer, deproteinised, and RNA was precipitated with 2 vol. 95% ethanol. Nuclear RNA samples dissolved in 200 μ l NETS buffer were centrifuged in 4 ml linear 15-30% sucrose density gradients in NETS buffer at 23 °C for 90 min at 56,000 r.p.m. in a Beckman SW56 rotor. Cytoplasmic RNA dissolved in 1 ml NETS buffer were centrifuged in 36 ml SDS-containing 15-30% sucrose density gradients at 23 °C for 17 h at 23,000 r.p.m. in a Beckman SW27 rotor. Radioactivity in 45S, 32S, 28S, 18S RNA were expressed as a percentage of the total acid-insoluble radioactivity in the gradient, including the pellets. In these analyses, 32S and 36S pre-rRNAs were combined since the distribution of these RNA molecules can vary in *ts 422E* cells²¹.

28S rRNA that appears in *ts 422E* cytoplasm at 39 °C is markedly methyl deficient.

In a precursor-product relationship, a reduced rate of conversion of intermediate species results in an increased proportion of precursor. If both wild-type and mutant cells are grown at 39 °C, inhibition of 45S processing in *ts 422E* should result in apparent nuclear accumulation of precursor molecules in the temperature-sensitive variants. This prediction was tested in the experiment shown in Fig. 1 where both cell types were labelled with ³H-uridine at the non-permissive temperature and the kinetics of accumulation of nuclear pre-rRNA and cytoplasmic rRNA were followed.

The cells were labelled in growth medium supplemented with unlabelled uridine to ensure that the specific activity of the UTP pool would be constant throughout the period of incorporation (unpublished data). In BHK cells, 45S and 32S pre-rRNAs and 28S and 18S rRNAs exhibited accumulation patterns consistent with their known precursor-product relationship^{7,8} (Fig. 1). Accumulation of 45S pre-rRNA in the mutant was, however, aberrant. During the first 30 min of labelling, the content of nuclear 45S RNA in *ts 422E* was 30–35% of the total labelled RNA compared with a

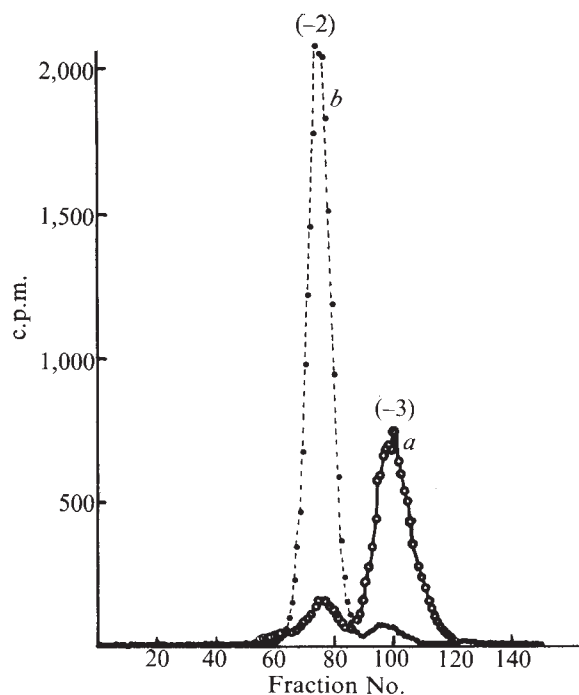


Fig. 2 Separation of mononucleotides and dinucleotides by DEAE-Sephadex chromatography. BHK cells were labelled for 3.5 h with (a) 20 $\mu\text{Ci ml}^{-1}$ L-methyl-³H-methionine (6.3 Ci mmol⁻¹) and (b) 0.1 $\mu\text{Ci ml}^{-1}$ ¹⁴C-uridine (57 mCi mmol⁻¹) in DME containing 20 μM methionine, 4 μM uridine, 20 mM sodium formate, and 20 μM guanosine to suppress non-methyl purine ring labelling¹² beginning 16 h after shift up to 39 °C. Ribosomal RNA prepared from MgCl₂-precipitated cytoplasmic RNP was sedimented at 23 °C in a 36 ml SDS-containing 15–30% sucrose gradient for 17 h at 23,000 r.p.m. in an SW27 rotor. 18S rRNA was precipitated from the gradient with 2 vol 95% ethanol, dissolved in 2 ml 0.3 N KOH, and hydrolysed for 18 h at 37 °C. The alkaline hydrolysate was neutralised with ice-cold 0.3 N perchloric acid, chilled at 0 °C for 10 min, and centrifuged at 5,000 r.p.m. for 5 min to remove precipitated potassium perchlorate. The neutralised sample was applied to a column of DEAE-Sephadex (19 $\times$ 1.2 cm; A-25, Pharmacia Fine Chemicals, Piscataway, New Jersey), washed into the column with 25 ml 20 mM Tris-HCl (pH 7.4), 7 M urea, 0.1 M NaCl, and eluted with a linear 100 ml gradient of 0.1–0.4 M NaCl in 20 mM Tris-HCl (pH 7.4), and 7 M urea^{10,22}. After completion of the gradient, the column was washed with 40 ml 0.4 M NaCl, 20 mM Tris-HCl (pH 7.4), and 7 M urea. Fractions were assayed for radioactivity by counting in a gel of 2.0 ml H₂O plus 10 ml scintillation fluid containing 2 parts xylene, 1 part Triton X-100 and 8 g Omnifluor (New England Nuclear, Boston, Massachusetts) per litre.

maximum of only 15–20% in BHK cells in similar conditions (Fig. 1). As stable nuclear and nucleolar RNAs accumulated, the fraction of radioactivity in 45S pre-rRNA decreased until by 2–3 h the relative amount of pre-rRNA was the same in the mutant and wild-type cells. The kinetics of mature cytoplasmic 18S and 28S rRNA accumulation in the mutant showed, as before⁹, a marked inhibition of new 28S rRNA appearance at 39 °C. The early increase in nuclear 45S pre-rRNA content in the mutant indicated an expansion of the nucleolar pool of 45S pre-rRNA, suggesting that the transcript was deficient in some essential aspect.

Because methylation of 45S pre-rRNA is an apparent requirement for completion of ribosome maturation^{1,9}, we compared the extent of 45S methylation in the wild-type and mutant cells. Ninety-five per cent of methylations found in HeLa cell 45S pre-rRNA are modifications of the 2'-OH ribose moiety of the nucleoside¹. Since this modification protects the phosphodiester linkage between the 3'-hydroxyl of that nucleotide and the 5'-hydroxyl of the neighbouring nucleotide from alkaline hydrolysis, separation of mononucleotides from dinucleotides on anion exchange columns¹⁰ after alkaline hydrolysis provides an estimate of rRNA methylation (Fig. 2). As shown in Fig. 2, approximately 96% of the uridine incorporated into 18S rRNA is found in the mononucleotide fraction, but methyl label is preferentially incorporated into the dinucleotides. This pattern demonstrates the general labelling characteristics of ³H-uridine and the relative specificity of methyl-³H-methionine for incorporation into 2'-hydroxyl ribose residues.

The relative methylation of pre-rRNA in BHK and *ts 422E* cell lines was examined initially by pulse labelling at 39 °C with methyl-³H-methionine and ¹⁴C-uridine (Table 1). The ratio of ³H-methyl/¹⁴C-uridine radioactivity was used as an index of the extent of methylation. By this criterion, 45S pre-rRNA from the mutant is approximately 50% undermethylated, and the 32S RNA has only a third of the methyl content of wild-type 32S RNA at the non-permissive temperature. Since 28S rRNA in mammalian cells is derived from the 32S molecule^{11,12}, undermethylation of 28S sequences would result in 32S RNA exhibiting a lower degree of methylation than complete 45S molecules which also contain properly methylated 18S RNA sequences (Table 1).

The apparent undermethylation observed in *ts 422E* at 39 °C could also be explained by decreased uptake of methionine relative to uridine. Therefore, we estimated ribose methylation of precursor and mature ribosomal RNA in BHK and *ts 422E* cells by measuring radioactivity in alkali-resistant dinucleotides of uridine-labelled cells. Although this approach measured only 2'-O-methylribose in or adjacent to uridine and/or cytidine, difficulties resulting from possible pool and transport differences were eliminated. The extent of undermethylation would seem less by

Table 1 Relative extents of methylation at 39 °C in BHK and *ts 422E* pre-rRNA

RNA	Radioactivity (c.p.m.)		
	³ H-methyl	¹⁴ C-uridine	³ H-methyl/ ¹⁴ C-uridine
BHK 45S	2,230	8,034	0.28
422E 45S	3,407	21,689	0.16
BHK 32S	5,115	28,611	0.18
422E 32S	5,717	98,716	0.06

BHK and *ts 422E* cells were labelled 45 min with 25 $\mu\text{Ci ml}^{-1}$ L-methyl-³H-methionine and 0.1 $\mu\text{Ci ml}^{-1}$ ¹⁴C-uridine as in Fig. 2, beginning 18 h after shifting to 39 °C. Nucleoli prepared as described by Penman²³ were deproteinised (Fig. 1) and nucleolar RNA was centrifuged in 36 ml linear 15–30% sucrose density gradients in NETS at 20,000 r.p.m. for 16 h at 23 °C in an SW27 rotor. Aliquots (50 μl) of gradient fractions were assayed for acid-insoluble radioactivity. Nominal 45S RNA and 32S RNA peak fractions were precipitated with ethanol, and chromatographed on DEAE-Sephadex after alkaline hydrolysis as in Fig. 2. Values represent total combined radioactivity in the mononucleotide and dinucleotide peaks.

Table 2 Estimation of 2'-O-methyl ribose in uridine-labelled ribosomal pre-rRNA

	Radioactivity Mononucleotides (-2) (c.p.m. $\times 10^{-4}$)		Radioactivity Dinucleotides (-3) (c.p.m. $\times 10^{-3}$)		Total radioactivity in dinucleotides (%)	
	33 °C	39 °C	33 °C	39 °C	33 °C	39 °C
Experiment 1						
BHK 45S	—	60.0	—	8.3	—	1.36
422E 45S	—	53.3	—	4.8	—	0.89
Experiment 2						
BHK 45S + 32S	75.6	105.1	18.1	24.7	2.33	2.34
422E 45S + 32S	252.8	57.9	56.9	11.9	2.20	1.75
BHK 28S	82.1	70.7	23.1	19.5	2.73	2.69
422E 28S	112.0	7.7	31.3	1.0	2.55	1.32
BHK 18S	67.6	25.5	25.3	11.9	3.60	4.47
422E 18S	115.1	38.2	57.0	17.1	4.70	4.30

In experiment 1, BHK and *ts* 422E cells were pulse labelled in DME with 100 $\mu\text{Ci ml}^{-1}$ ^3H -uridine beginning 22 h after shift-up to 39 °C. 45S pre-rRNA prepared by sucrose density gradient sedimentation of nucleolar RNA was hydrolysed with 0.3 N KOH at 37 °C and chromatographed on DEAE-Sephadex (Table 1). In experiment 2, wild-type and mutant cells were labelled 3.5 h with 5 $\mu\text{Ci ml}^{-1}$ ^{14}C -uridine at 33 °C. 45S and 32S nucleolar RNA was prepared and analysed as described above. MgCl_2 -precipitated cytoplasmic RNP was deproteinised and RNA was sedimented in sucrose density gradients as in Fig. 1, and the major fractions under the separated 28S and 18S rRNA peaks were pooled and precipitated with 2 vol 95% ethanol. Contaminating poly(A)-containing mRNA was removed by oligo(dT)-cellulose chromatography²⁵. Purified rRNAs hydrolysed with KOH were chromatographed on DEAE-Sephadex. Details of experiment 3 are the same as for experiment 2, except that wild-type and mutant cells were labelled beginning 19 h after shift-up to 39 °C. Three replicate samples of uridine-labelled HeLa cell rRNA were hydrolysed and chromatographed on DEAE-Sephadex to test the reproducibility of the technique. For these replicates the mean fraction of ^3H -uridine in the dinucleotide region was 2.83% with a standard deviation of 0.096 or 3.4% of the mean value.

this assay, because these experiments only scored for uridine label in dinucleotides against mononucleotides.

The results of DEAE-Sephadex chromatography of alkali-hydrolysed RNA from cells labelled for 30 min or 3.5 h with uridine (Table 2) confirmed the previously observed undermethylation of *ts* 422E nucleolar and 28S cytoplasmic rRNA. With the exception of 18S rRNA which yielded less reproducible values than other species of RNA, the differences between mutant and wild-type cells seen at the permissive temperature can be attributed to variation in the method (see legend to Table 2), but the differences observed at 39 °C must reflect the conditional lethal phenotype. The similarity of RNA methylation in the wild-type cells at 33 and 39 °C is a further indication of the accuracy of this analysis (Table 2).

Methylation of rRNA is of two types: 2'-O-ribose modification and methylation of specific sites in the purine and pyrimidine rings. In HeLa cells, 80–90% of methyl derivatives in rRNA are modifications of the 2-OH-ribose moiety^{1,13,14}. The experiments presented above clearly show deficient methylation of 2'-O-ribose moieties in 28S rRNA and its precursors in *ts* 422E cells grown and labelled at 39 °C. Since phosphodiester bonds adjacent to base methyl derivatives may also be resistant to alkaline hydrolysis¹³, however, the observed differences may not be due exclusively to alterations in 2'-O-methylation.

Physical studies have shown that methylation of ribose residues can alter the conformation of single-stranded RNA molecules¹⁶. Although the details of eukaryotic ribosome maturation are not known, it is likely that processing of 28S rRNA requires the sequential association of specific proteins and 32S RNA to give rise to the nucleolar 55S pre-ribosomal particles. Inadequate methylation of the 28S segment could alter the conformation of the ribosomal precursors, preventing the normal association of processing enzymes and thus inhibiting the appearance of 28S RNA in the cytoplasm. Assuming 18S RNA sequences at the 5' end of the transcript¹⁷ are properly methylated, and that methylation and transcription are closely coupled, it is possible that the distal portions of the transcript containing 28S sequences are inhibited from being adequately modified. Alternatively, a precursor-associated protein may be the primary target of the temperature-sensitive lesion, placing the transcript in a conformation within the precursor particle that is unfavourable for completion of adequate methylation.

After submission of this paper, Toniolo and Basilico

reported increased accumulation of nucleolar pre-rRNA in *ts* 422E at 39 °C compared with wild-type cells, but these authors did not detect differences in extent of pre-rRNA methylation²⁵. Our assay for rRNA methylation may have been more specific, however, since we labelled with methionine in conditions which suppressed non-methyl purine ring labelling (see legend to Fig. 2).

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Restriction of template capacity of rat liver chromatin by a non-histone peptide from calf thymus

WE have reported¹ the isolation of a thymic peptide which strongly inhibits DNA-directed RNA polymerase *in vitro*. We have shown that transcription is controlled at the level of initiation. The active factor was isolated from aqueous ultrafiltered thymus extracts, purified by chromatography, first on DEAE-cellulose and then on Dowex 50 WX2, and characterised as a peptide of low molecular weight (<5,000). Its biological activity cannot be attributed to the presence of a nuclease or of a histone fragment. The inhibition of RNA synthesis is due to an ionic interaction between the peptide and DNA. The physicochemical and thermodynamic properties of this interaction are described elsewhere². We report here the effect(s) of the thymic peptide on transcription of rat liver chromatin. With some obvious limitations³, the *in vitro* chromatin transcription system is closer to the *in vivo* state than the purified DNA. Our experiments were prompted by our previous results, which demonstrated that the thymic extract modifies the physicochemical state of DNA and the interactions between DNA and nuclear proteins in the liver of old rats^{4,5}.

The purified thymic fraction, designated fraction 1₆ (see legend to Fig. 1), strongly inhibited chromatin transcription by *Escherichia coli* RNA polymerase (Fig. 1). This effect was proportional to the concentration of factor up to 70% inhibition. Contrary to our data on inhibition of DNA transcription¹, even when doses of the factor larger than shown in Fig. 1 were added, chromatin transcription was never completely stopped, indicating that the thymic factor affected only part of the RNA synthetic pathways. Since the thymic factor acts on initiation of transcription rather than on elongation¹, it is possible that the peptide cannot inhibit chromatin transcription by endogenous RNA polymerases, which are probably linked to the chromatin itself. We therefore tested for chromatin-directed RNA synthesis *in vitro* without added *E. coli* polymerase, in both the presence and absence of the thymic extract. Figure 2a shows that, in the absence of exogenous RNA polymerase, chromatin transcription constituted only 10–15% of total transcription observed in the presence of exogenous RNA polymerase from *E. coli*. In these conditions the thymic factor was ineffective. These data support the hypothesis that the thymic factor exerts its activity on initiation.

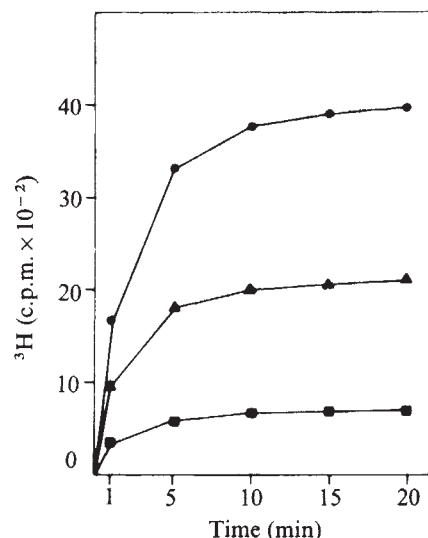


Fig. 1 Time course of RNA synthesis directed by rat liver chromatin: incorporation of ³H-CMP. 0.75 ml of the complete incubation mixture for RNA synthesis contained: 30 µmol Tris buffer (pH 7.5), 3 µmol MgCl₂, 0.75 µmol MnCl₂, 9 µmol β-mercaptoethanol, 0.3 µmol each of ATP, GTP, CTP and UTP, 2 nmol ³H-CTP (22 Ci nmol⁻¹, Radiochemical Centre, Amersham), 4 U RNA polymerase from *E. coli* and 10 µg chromatin DNA. 0.1 or 0.2 ml of the purified thymic fraction corresponding respectively to 4 µg and 8 µg protein was added to the incubation mixture; in the controls the extract was replaced by 0.1–0.2 ml saline. Incubation for RNA synthesis was carried out in 10-ml Erlenmeyer flasks at 37 °C, 75 shakes per min. Determinations of the radioactivity incorporated into the acid-insoluble fraction of RNA, the preparation of the thymus extracts and the purification of the active factor, designated fraction 1₆, were carried out as before¹. Fraction 1₆ consisted of peptide(s) of low molecular weight: thin layer high voltage electrophoresis showed four ninhydrin-positive bands. As far as the affinity binding between DNA and thymus factor is concerned, evidence presented elsewhere² indicates that when DNA at 1 mg ml⁻¹ is incubated with a fraction 1₆ (100 µg ml⁻¹), about 20 µg protein binds to DNA. This is taken as evidence that fraction 1₆ is mostly composed of active thymic factor. Total chromatin was extracted from livers of 12-month-old Sprague-Dawley rats according to the modified method of Bostock and Prescott¹²; nuclei were not broken by sonication but by homogenisation twice in Potter Helvehym after rapid freezing and thawing. We modified Bostock and Prescott's method to obtain the chromatin in the most native state possible. The RNA polymerase from *E. coli* was purified as described before¹³. The data in the experiments described are the average of experiments made in duplicate, the values of which have never shown variations above 10%. ●, Control; ▲, fraction 1₆: 5 µg protein per ml (final concentration); ■, 10 µg protein per ml (final concentration).

Table 1 Template capacity of calf thymus DNA and of rat liver heterochromatin and euchromatin

Reaction mixture		³ H-CMP incorporation (c.p.m. mean ± s.e.m.)		% Inhibition
		Control	Thymus factor added	
Calf thymus DNA	+ <i>E. coli</i> RNA polymerase	22,015 ± 810	1,601 ± 245	93
Calf thymus DNA	+ calf thymus RNA polymerase	20,242 ± 640	1,721 ± 315	91
Rat liver heterochromatin	+ <i>E. coli</i> RNA polymerase	2,570 ± 180	960 ± 105	63
Rat liver euchromatin	+ <i>E. coli</i> RNA polymerase	4,015 ± 215	1,120 ± 148	72
Rat liver heterochromatin	+ calf thymus RNA polymerase	1,137 ± 110	653 ± 80	42
Rat liver euchromatin	+ calf thymus RNA polymerase	1,701 ± 98	855 ± 60	49
Rat liver heterochromatin	+ rat liver RNA polymerase	1,580 ± 124	615 ± 132	61
Rat liver euchromatin	+ rat liver RNA polymerase	2,424 ± 170	715 ± 97	70

Incorporation of ³H-CMP by means of RNA polymerase from *E. coli*, calf thymus or rat liver, in both the presence and absence of the thymic factor (final concentration 10 µg protein per ml), after 30 min of incubation. The preparation of thymus extract, the incubation mixture and other conditions for RNA synthesis were as in the legend to Fig. 1. RNA polymerase from *E. coli* was purified as described by Hurwitz¹³ and the RNA polymerases II from calf thymus and rat liver as described by Stein and Hausen¹⁴. The extraction of the chromatin from rat liver and the fractionation of the total chromatin in heterochromatin and euchromatin were carried out according to the modified method (see legend to Fig. 1) of Bostock and Prescott¹². In some experiments the fractionation of the total chromatin in heterochromatin and euchromatin was carried out by means of gel filtration on BioGel A-50 (BioRad) as described by Janowski *et al.*¹⁵. The determinations of the buoyant density and of the DNA, RNA and protein contents of hetero- and euchromatin were effected by means of the determination of the refraction index¹⁶ and of diphenylamine, orcinol and Lowry reactions respectively:

	Buoyant density	DNA	RNA	Protein
Heterochromatin	1.711	1	0.160	2.071
Euchromatin	1.699	1	0.175	1.714

To confirm this hypothesis, chromatin was preincubated for 1 min in the presence of *E. coli* RNA polymerase and UTP, GTP and ATP nucleotides. At time zero the reaction mixture was made 0.4 M in ammonium sulphate, the thymic fraction or saline was added and the propagation reaction was started by adding CTP and ^3H -CTP. During preincubation polymerase binds to chromatin and initiates transcription, but propagation is blocked by the absence of CTP: when CTP is added propagation proceeds, but the presence of high concentrations of ammonium sulphate precludes further initiation or reinitiation^{6,7}. Figure 2b shows that in these conditions the thymic factor did not affect RNA synthesis and therefore inhibited the initiation reaction of transcription. Later we investigated the action of the thymic factor on transcription in different states of chromatin condensation: that is heterochromatin and euchromatin, the characteristics of which are given in the legend to Table 1. The transcription of heterochromatin and euchromatin, in both the absence and presence of thymic factor, was tested by adding various RNA polymerases: different specificities of chromatin transcription by RNA polymerase from prokaryotic or eukaryotic cells have been reported^{8,9}. RNA polymerases from *E. coli* and RNA polymerases II from rat liver and calf thymus were used. Table 1 shows that the thymic peptide inhibited transcription of heterochromatin and euchromatin by polymerases from both prokaryotic and eukaryotic cells, regardless of the organ of origin of eukaryotic polymerases.

The data presented here provide evidence for our hypothesis that the thymic peptide is responsible for regulation at the level of transcription. The addition of the factor restricts the distribution of available sites for the initiation of transcription on the chromatin. These observations

suggest that the factor regulates transcriptional capacity, which may be related to cell differentiation. This is supported by our finding that the thymic factor was not extracted from differentiated tissues such as kidney, whereas it was particularly abundant in bull spermatozoa (unpublished results). The inhibition of chromatin transcription by this factor extracted from spermatozoa may explain our previous data¹⁰ showing that extracts of spermatozoa inhibit differentiation of seminiferous tubule cells of testicles from young rabbits.

The data presented here are in good agreement with the demonstration¹¹ that chromatin from several mammalian species contains non-histone protein fractions including specific negative regulatory factors ionically linked to DNA.

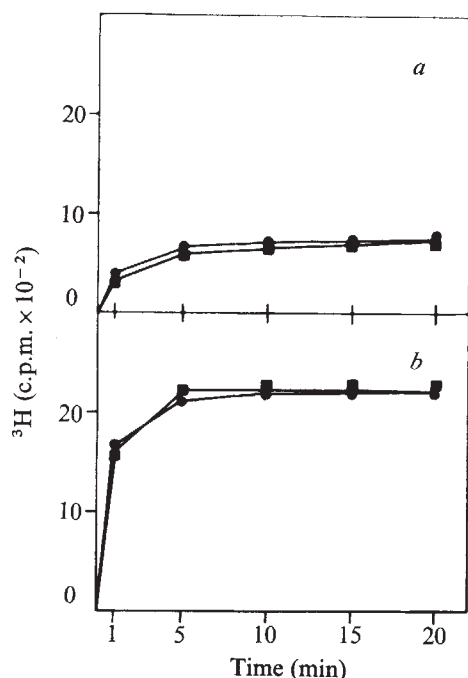
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Fig. 2 Time course of RNA synthesis directed by rat liver chromatin: incorporation of ^3H -CMP. *a*, In the absence of exogenous *E. coli* RNA polymerase in the incubation mixture. Other ingredients for RNA synthesis were as given in the legend to Fig. 1. ●, Control; ■, thymic fraction 1₆ (final concentration 10 μg protein per ml). *b*, The chromatin was preincubated for 1 min in the presence of *E. coli* RNA polymerase and ATP, GTP and UTP. At time zero the reaction mixture was made 0.4 M in ammonium sulphate, thymic factor or saline was added and the propagation reaction was started by adding CTP and ^3H -CTP. Incubation mixture and other conditions for RNA synthesis were as given in the legend to Fig. 1. ●, Control; ■, fraction 1₆ (final concentration 10 μg protein per ml).



Independence of plasmid incompatibility and replication control functions in *Staphylococcus aureus*

THERE are two basic requirements for the stable maintenance of an autonomous plasmid replicon, namely replication at a constant rate with respect to the cell division cycle and distribution of replicas to daughter cells.

The precise nature of the genetic functions involved in satisfying these two requirements has yet to be ascertained; but it is probable that in general, both plasmid-linked and host chromosome-linked genetic determinants are involved. Of the former there are two well defined functional classes: (1) genes involved directly in replication¹⁻³ and its control, including the origin, and (2) genes involved in the determination of plasmid incompatibility specificity⁴⁻⁶. No genes known to affect distribution of plasmid replicas have been identified.

Incompatibility, the inability of closely related plasmids to be stably co-maintained⁷, has been found for the penicillinase plasmids of *S. aureus* to be determined by a genetic locus, *inc*, that has not been separable by recombination from *repA*, a gene encoding a diffusible product required for autonomous replication^{1,4}. Together, these two genetic functions plus the origin define a local region of the plasmid that is both necessary and sufficient for autonomy, the *mcr* region⁴. Similar localisation of autonomy functions has been observed for plasmids of enteric organisms⁸.

Because the replication of a plasmid entering a cell carrying another with which it is incompatible is temporarily or, possibly in some cases permanently, inhibited⁹⁻¹¹, it has been

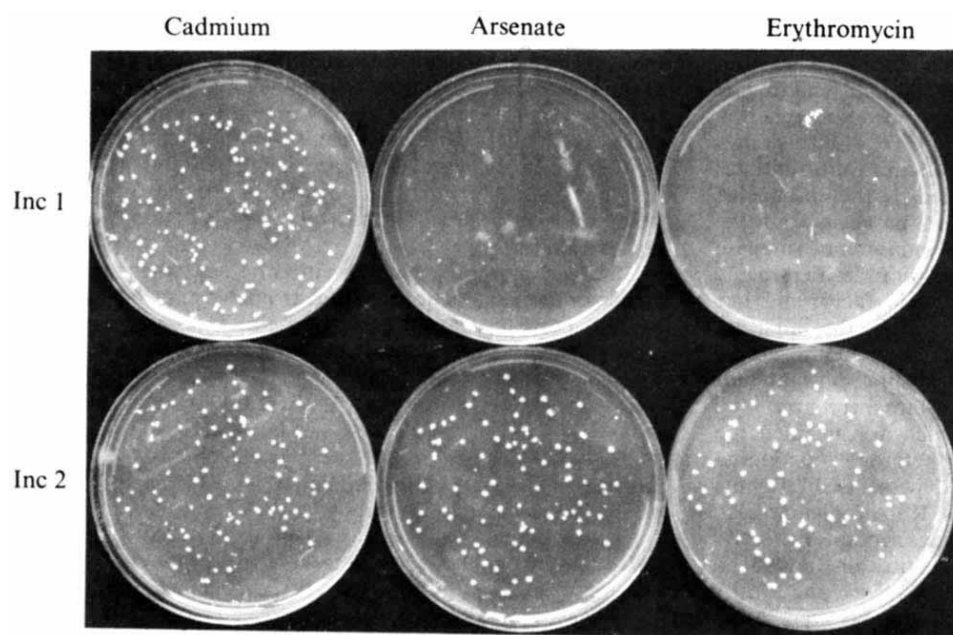


Fig. 1 Incompatibility between integrated and autonomous plasmids. Cultures of RN2428 (upper part of photograph) and RN2429 were diluted and plated on non-selective medium and incubated at 43 °C. The resulting colonies were replica-plated to three different selective media: GL-cadmium agar (selective for the integrated plasmid, pRN3091); GL-arsenate; and GL-erythromycin (selective for the autonomous plasmid, pRN3025 and pRN4031). The replicas were photographed after 18 h of incubation at 32 °C.

assumed that the incompatibility determinant is involved in replication control, for example, it may encode a specific repressor of replication¹².

In this report we present evidence suggesting that in the *S. aureus* system, the plasmid *inc* and *repA* determinants are genetically as well as functionally distinct and that the *inc* locus is not involved in replication or its control but instead may be involved in the distribution of replicas.

Two different chromosomally integrated derivatives of an incompatibility type 1 (Inc1) *S. aureus* penicillinase plasmid, pI258, were studied. One of these, pRN3150, is incompatibility-defective, yet expresses the *repA* function¹; the other, pRN3091, is *repA*⁻ (thermosensitive for replication) yet expresses incompatibility at the restrictive as well as at the permissive temperature. Strains carrying the *repA*⁺ *inc*⁻ pRN3150 were able to maintain stably and complement for replication seven different superinfecting *repA*⁻ Inc1 plasmids¹. But a recombinant derivative, pRN3151, in which the *repA36* allele had been substituted for the *repA*⁺, was unable to complement for replication any of the seven *rep*⁻ mutants (unpublished data); thus, there is evidence for only one plasmid-linked *rep* cistron.

The converse test, namely whether a *repA*⁻ mutant can

express incompatibility or not was also performed with an integrated plasmid (in this case the *Rep*⁻(Ts) pRN3091¹³) since there is no simple way to test an *autonomous Rep*⁻(Ts) plasmid for the expression of incompatibility at the restrictive temperature. Transductional superinfection of strain RN1979 (carrying the integrated pRN3091) with plasmids pRN3025 (Inc1) and pRN4031 (Inc2) was accomplished by selection for erythromycin resistance¹⁴. Eight transductants from each cross were examined and the following results were obtained: as expected, the Inc2 plasmid, pRN4031, was always maintained stably without selection. The Inc1 plasmid, pRN3025, could also be maintained but only by continued selection for erythromycin or arsenate resistance. Organisms grown on arsenate-containing medium, selective for the donor plasmid, were diluted and plated on non-selective medium (GL agar⁶) and allowed to form colonies at 32 °C and at 43 °C (the restrictive temperature for the *Rep*⁻(Ts) mutation). These were then replicated separately to plates containing cadmium, arsenate, or erythromycin. As shown in Fig. 1, colonies of RN2428 (RN1979 superinfected with the *inc1* plasmid, pRN3025) lost arsenate and erythromycin resistance during growth at 43 °C on non-selective medium, whereas colonies of

Table 1 Staphylococcal strains and plasmids*

a Plasmids		Genotype	Derivation
Plasmid number			
pI258	<i>bla</i> ⁺ <i>asa</i> ⁺ <i>asi</i> ⁺ <i>ermB</i> ⁺ <i>inc1</i> ⁺ <i>mer</i> ⁺ <i>rep</i> ⁺ <i>cadA</i> ⁺ <i>bis</i> ⁺ <i>lea</i> ⁺		Naturally-occurring
pII147	<i>bla</i> ⁺ <i>asa</i> ⁺ <i>inc2</i> ⁺ <i>rep</i> ⁺ <i>mer</i> ⁺ <i>cadB</i> ⁺ <i>bis</i> ⁺ <i>lea</i> ⁺ <i>cadA</i> ⁺		Naturally-occurring
pRN3025	pI258 <i>bla</i> -401 <i>cadA52</i> <i>ermB</i> ⁺		Mutation of pI258
pRN3032	pI258 <i>bla</i> -401 <i>cadA52</i> <i>mer</i> -14 <i>repA36</i>		Mutation of pI258
pRN3091	pI258 <i>bla</i> 1443 <i>asa</i> -33 <i>ermB20</i> <i>repA18</i>		Mutation of pI258
pRN3150	pI258 <i>bla</i> 1443 <i>asa</i> -33 <i>ermB18</i> <i>inc1</i> ⁻ <i>repA</i> ⁺		Integration of pI258 mutant
pRN3151	pI258 <i>bla</i> 1443 <i>asa</i> -33 <i>ermB18</i> <i>inc1</i> ⁻ <i>repA36</i>		Recombinant pRN3150 × pRN3032
pRN4031	pII147[<i>inc2</i> ⁺ <i>cadB</i> ⁺ <i>bis</i> ⁺ <i>lea</i> ⁺]; pI258[<i>bla</i> -401 <i>asa</i> ⁺ <i>asi</i> ⁺ <i>ermB</i> ⁺] <i>mer</i> ⁺ <i>cadA11</i>		Recombinant pII147 × pI258
b Strains†		Plasmids contained	Reference
Strain number			
RN325		pRN4031	
RN557		pRN3025	
RN1500		Ω42[pRN3150]	ref. 1
RN1974		Ω42[pRN3151]	This report
RN1979		Ω50[pRN3091]	ref. 13
RN2428		Ω50[pRN3091]; pRN3025	This report
RN2429		Ω50[pRN3091]; pRN4031	This report

* Gene abbreviations and other nomenclature are as recommended by Novick *et al.*⁷.

† The host bacterium is strain 8325-4, except for RN1974 in which the host is strain 147 (ref. 14).

RN2429 did not. Similar results were obtained for colonies of these two strains grown at 32 °C. These results are interpreted as the expression of incompatibility by the integrated *repA*⁻ plasmid at both permissive and restrictive temperatures, demonstrating the independence of the *inc* and *repA* determinants.

To verify the inference that loss of arsenate and erythromycin resistance is an indication of loss of the entire plasmid, thirty colonies of strain RN2428 that had been grown on non-selective medium were scored by disk sensitivity tests and by the PNCB stain for penicillinase activity¹⁴. There were scorable allelic differences at four plasmid loci—*bla*, *asa*, *cad*, and *erm*; on subculture, twenty-eight of the colonies showed the same resistance pattern as the resident plasmid and a penicillinase stain pattern indicative of homozygosity for the integrated *bla* locus. Two colonies showed stable patterns suggestive of recombination between the integrated and autonomous plasmids.

Although it seemed likely on the basis of the foregoing results that the superinfecting penicillinase plasmid could replicate in the autonomous state, it seemed important to attempt to demonstrate this replication directly and to assess it quantitatively. Accordingly, a dye-buoyant density gradient analysis of plasmid replication was done¹, and the results shown in Fig. 2 were obtained. Incorporation of thymidine into circular duplex plasmid DNA by the incompatible heterozygote, RN2428 (Fig. 2a) was nearly as extensive as that by the compatible heterozygote RN2429 (Fig. 2b), by comparison with strains carrying the autonomous plasmid alone in each case. As this result could have been due to the presence of unsuspected recombinants, the cultures were tested at the end of the labelling period for the proportion of organisms that would still lose the superinfecting plasmid during incubation on non-selective media. Accordingly, a replica-plating test similar to that shown in Fig. 1 was performed and showed that approximately 80% of the organisms in the RN2428 culture were still arsenate-resistant and that at least 95% of these lost resistance on incubation on non-selective medium. On the other hand, 95% of the organisms in the RN2429 culture were arsenate-resistant and of these, less than 1% lost arsenate resistance during incubation on non-selective medium.

It is possible that the somewhat reduced incorporation into plasmid DNA in strain RN2428 (see Fig. 2a) was due to a reduction in the number of autonomous copies of the

plasmid, normally 8 to 12 per cell¹⁵, in that strain. Experiments are in progress to test this possibility.

We conclude from these experiments that the known plasmid loci affecting incompatibility and replication seem to be genetically separate and functionally distinct in *S. aureus* and that in this system, the expression of incompatibility does not involve inhibition of replication.

Consistent with this conclusion is the fact that it is generally possible, both in *S. aureus* and in *E. coli*, to construct strains carrying pairs of incompatible autonomous plasmids. In such heterozygotes, which can be maintained only by double selection, the two incompatible plasmids replicate side-by-side, and segregate at a measurable, constant rate on nonselective media^{9,16,17}. We should like to propose, therefore, a model in which incompatibility between autonomous plasmids is an automatic consequence of the partition process. This model would apply to plasmids with random partition as well as to those with equipartition. Our primary formulation is, however, for the latter since equipartition is evidently a property of staphylococcal plasmids¹.

(1) Copies of a given plasmid are partitioned at cell division by transient attachment to a "partition structure" (the membrane?—compare Jacob *et al.*²).

(2) The partition and replication control mechanisms are incompatibility-type specific; that is, they cannot distinguish among plasmids belonging to the same incompatibility set (but they can distinguish among members of different sets).

(3) Plasmids belonging to the same incompatibility set are randomised between cell divisions so that there is a high probability that gross disproportion will develop among them during cell division.

(4) Any disproportion generated during division is likely to be maintained by replication during the next interdivision period; such disproportions will then be amplified during subsequent divisions until subclones containing only one of the pair are generated.

Note that the behaviour of homogenic incompatible plasmids illuminates a feature of equipartition that would otherwise remain obscure, namely randomisation of the plasmid copy pool. The crux of the model is that disproportions generated by this randomisation cannot be corrected because their existence cannot be recognised. The model makes a number of predictions that are readily testable: (1) the segregation behaviour of homogenic

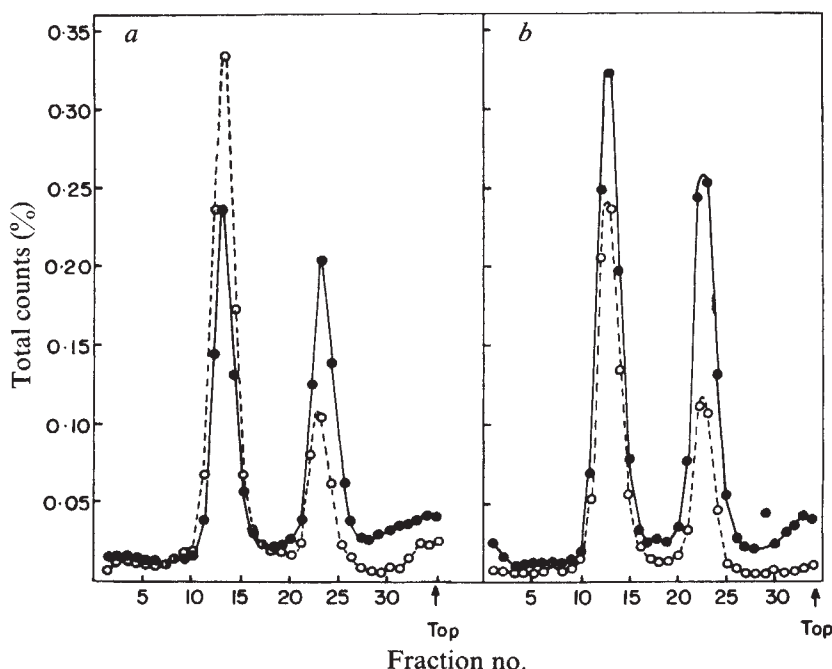


Fig. 2 Replication of incompatible plasmids. *a*. A cleared lysate¹⁸ of a ³H-labelled RN2428 culture mixed with a ¹⁴C-labelled RN557 culture was centrifuged to equilibrium in an ethidium bromide-caesium chloride gradient which was fractionated and counted by standard procedures. Counts were normalised to total incorporation into the respective cultures. *b*. Dye-buoyant density gradient pattern for lysate of a mixture of a ³H-labelled RN2429 culture and ¹⁴C-labelled RN325. For both, dashed curve, ¹⁴C; solid curve, ³H. Density increases from right to left.

incompatible plasmid heterozygotes is solely a function of copy numbers and can be predicted by simple combinatorial analysis; (2) the incompatibility locus is a recognition site for partition and so does not encode a protein—thus it may be regarded as a prokaryotic centromere analogue; (3) a (membrane) protein exists that recognises and binds to this site—such a protein could either be plasmid- or host-coded; (4) the incompatibility locus is not essential for autonomous replication; (5) plasmids with absent or defective *inc* loci will be partitioned randomly in the absence of other plasmids; (6) paradoxically, *Inc*⁻ plasmids will not be compatible with *Inc*⁺ plasmids of the same original class.

It should be pointed out that this model as stated deals with the hereditary consequences of only one of several possible combinations of replication and partition specificities. For example, it is implicit in prediction 6 (above) that equipartition is not a prerequisite for incompatibility; so long as the replication system cannot distinguish between plasmids, the inevitable disproportions arising during cell division will not be correctable and the result will be segregation. In such cases, the incompatibility locus will not be a centromere analogue but rather will correspond to the determinant(s) of replicon recognition specificity.

It should also be mentioned that there are two known situations in which the presence of one plasmid evidently inhibits the replication of another with which it is incompatible. These two situations are (1) delayed replication of an entering plasmid^{9,11} and (2) inhibition by a cointegrate replicon of the replication of an autonomous plasmid incompatible with the subsidiary component of the cointegrate. Two examples of the latter are, *Hfr* strains superinfected by *F* plasmids¹⁰ and a *colE1*-*pSC101* cointegrate superinfected by *pSC101*¹⁷. All of these situations may be manifestations of a copy number control mechanism. In case (1) above, the resident plasmid is present at its full complement of copies and so an entering one is disadvantaged for that reason. Eventually, however, the entering plasmid is able to establish and replicate. In case (2) involving cointegrates, if the number of copies of the subsidiary replicon (for example, integrated *F* or *pSC101*) is maintained automatically at a level equal to or greater than its normal autonomous multiplicity, one would expect replication of autonomous copies of the corresponding plasmid to be inhibited and the establishment of that plasmid to be prevented.

The above considerations suggest that as many as three different kinds of genetic mechanism, each operative under a particular set of conditions, may be manifested as plasmid incompatibility. These are (1) a recognition site for equipartition, (2) a recognition site for replication specificity, and (3) a copy number control mechanism.

The basis of the expression of incompatibility by the integrated *S. aureus* plasmid described here is not intuitively obvious from these considerations. It is unlikely that copy control is involved, since the autonomous plasmid (when alone) is normally present at 8–12 copies per cell¹⁵ and the integrated one at 2–4¹⁸. Presumably, therefore, the integrated plasmid somehow causes random partition of the autonomous one and prevents correction of the deficiencies in the copy number of the latter that consequently arise during division. Experimental analysis of this possibility is in progress.

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Low angle X-ray diffraction patterns of squid retina

THE structure of photoreceptor membranes in the rod outer segments of vertebrates has been studied using X-ray diffraction^{1–3}, and electron density profiles of the disk membrane at a resolution of 14 Å have been obtained⁴. The ultrastructure of invertebrate rhabdomeric visual cells have been studied using electron microscopy⁵ and birefringence⁶, but not X-ray methods. We report here low angle X-ray diffraction patterns recorded from squid retina after fixation in glutaraldehyde.

In the squid the rhabdomere structures of the retinula cells⁷ are contained within the distal segments⁸ (by analogy with the retinal rod outer segments of vertebrates). Electron micrographs^{7,8} of the distal segments of squid show an array of microvilli and show that the long axes of the microvilli are perpendicular to the light direction. A single microvillus^{7,8} is shown as a thin hollow cylinder about 1–2 µm long and about 700–1,000 Å in diameter. The microvilli appear to be in regular hexagonal arrays. A three-dimensional reconstruction of the squid distal segment⁷ shows that the adjacent hexagonal arrays of different retinula cells are approximately perpendicular to each other. The squid retina is well suited to X-ray study because the volume fraction of the microvilli in the squid retina is large when compared with other invertebrates. The distal segments are rather long, about 300 µm, and the distal segments of squid⁷ like those of octopus⁹ are closely packed together.

Fresh squids were obtained at Marine Biological Laboratory, Woods Hole. After dissection the squid eyes were immediately fixed in 6% glutaraldehyde in Tris buffer (pH 7.0) and stored in a refrigerator at about 4 °C. In the X-ray experiment a 1-mm strip was cut from the fixed eye and mounted in a specimen chamber. The X-ray procedures for recording diffraction from squid retina were very similar to those used in obtaining X-ray patterns from vertebrate retina^{1–3}. Low angle X-ray diffraction patterns were taken with the X-ray beam incident parallel to the retina surface, and incident perpendicular to the light direction and to the long axis of each distal segment. Exposure times varied from 1 h to 1 d using an Elliott rotating anode microfocussing X-ray generator and an optically focusing X-ray camera¹⁰.

Initially, slit collimation was used to explore whether low angle X-ray diffraction could be recorded from our X-ray specimens of squid retina. A series of diffraction orders from a lamellar repeating distance of $d = 620 \pm 10$ Å along the light

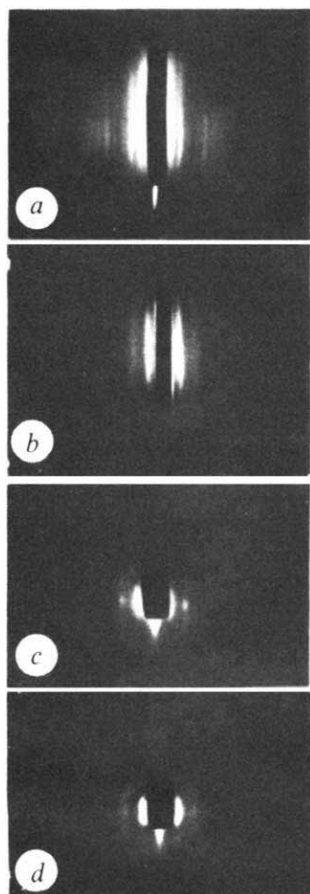


Fig. 1 *a*, Low angle X-ray diffraction pattern of squid retina after fixation in glutaraldehyde. The X-ray pattern was obtained using slit collimation and a specimen-to-film distance of 9 cm. Orders $h = 2$ to 8 of $d = 620 \text{ \AA}$ and a diffuse reflection centred at $d \sim 48 \text{ \AA}$ are visible. Scattering close to the beam stop obscures the first order of diffraction. *b*, Low angle X-ray diffraction pattern of squid retina after fixation in glutaraldehyde. The X-ray pattern was obtained using slit collimation and a specimen-to-film distance of 20 cm. The first two orders of $d = 620 \text{ \AA}$ are visible. The first order has a strong intensity, whereas the second is comparatively weak. *c*, Low angle X-ray diffraction pattern of squid retina after fixation in glutaraldehyde. The X-ray pattern was obtained using pinhole collimation and a specimen-to-film distance of 15 cm. The (01) and (10) reflections on the first layer line are off-meridional but appear to overlap as they have strong intensity. Three reflections are seen on the second layer line. The (11) reflection is on the meridian, whereas the (02) and (20) reflections are off-meridional. *d*, Low angle X-ray diffraction pattern of squid retina after fixation in glutaraldehyde. The X-ray pattern was obtained using pinhole collimation and a specimen-to-film distance of 15 cm. Less X-ray exposure was used to obtain this pattern. The (01) and (10) reflections on the first layer line are resolved as off-meridional reflections. The three reflections on the second layer line are also visible but faint.

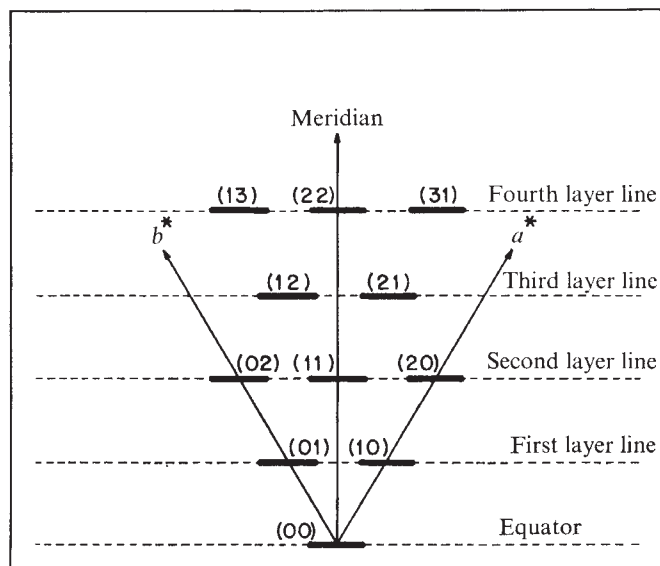
direction, was recorded. Generally, the first three orders of diffraction were discrete, whereas the higher orders merged together. When moderate exposure times were used the X-ray pattern extended out to a resolution of about $20 \text{ \AA}$. Occasionally, the first eight discrete orders could be seen. A low angle X-ray diffraction pattern, showing discrete orders $h = 2$ to 8 of a lamellar repeating distance of $d = 620 \text{ \AA}$ and a diffuse reflection centred at $d \sim 48 \text{ \AA}$, is reproduced in Fig. 1*a*. The first order of diffraction $h = 1$ is not resolved in Fig. 1*a* because of scattering close to the beam stop. In an X-ray experiment using less exposure time and an increased specimen-to-film distance of 20 cm the first two diffraction orders $h = 1$ and 2 of $d = 620 \text{ \AA}$ were recorded. This pattern is reproduced in Fig. 1*b*.

Unfortunately, the interpretation of the low angle X-ray patterns obtained using slit collimation is not straightforward.

The first step in interpreting the lamellar diffraction is to identify the orientation of the two-dimensional arrays relative to the light direction. The orientation of the arrays of microvilli is not easily obtained from a study of electron micrographs^{7,8}; and, so far, the orientation has not been established by electron microscopy. The recording of discrete orders of a lamellar repeat distance in the slit-collimated X-ray patterns indicates that the microvilli arrays have the same lattice dimensions and that they have the same orientation relative to the light direction. Thus it is important to verify whether the lattice is hexagonal, to establish the lattice dimensions and to determine the orientation of the microvilli arrays relative to the light direction.

The orientation of the two-dimensional arrays of microvilli has been determined by recording low angle X-ray diffraction patterns using pinhole collimation. The pinhole-collimated X-ray patterns require longer exposure times because of the weaker main beam and because of having to use a specimen-to-film distance of 15 cm to resolve diffraction orders along the layer lines. So far, only the first few diffraction orders have been recorded. Typical low angle X-ray diffraction patterns of squid retina are shown in Fig. 1*c* and *d*. The angle between the off-meridional reflections in our pinhole-collimated X-ray patterns is close to 60° ; and therefore it is assumed that the microvilli lattice is hexagonal. Note that the angle appears to be slightly less than 60° (it is about 55° in Fig. 1*c* and *d*), indicating that our X-ray specimens contain a microvilli lattice which is slightly compressed along the (1,1) direction. This slight compression probably arises as a result of sectioning and mounting the retina strip in the specimen chamber. The pinhole patterns do not reproduce well, but the off-meridional reflections (01) and (10) on the first layer line and the (11) meridional reflection and the weaker off-meridional reflections (02) and (20) on the second layer line can be seen in Fig. 1*c*. The off-meridional reflections (12) and (21) on the third layer line can be seen in the original negatives. In Fig. 1*c* the off-meridional reflections (01) and (10) are overexposed and tend to overlap in the reproduction. The pinhole pattern shown in Fig. 1*d* has less X-ray exposure and the (01) and (10) off-meridional reflections on the first layer are seen as discrete reflections. A drawing of the pinhole-collimated X-ray patterns

Fig. 2 A drawing of the two-dimensional microvilli lattice as shown by the pinhole collimated X-ray diffraction patterns. The light direction is along the meridian. The a^* and b^* axes of the hexagonal reciprocal lattice is indicated and the (11) direction is along the meridian. The reflections (01), (10), (11), (02), (20), (12) and (21) on the first three layer lines have been seen in a number of negatives. The reflections (13), (22) and (31) on the fourth layer line have not been recorded but are included for illustrative purposes. Note that this figure is rotated 90° relative to Fig. 1 so that the beam stop is along the equator.



is shown in Fig. 2. Note that Fig. 2 is rotated 90° relative to Fig. 1 so that the beam stop is along the equator. The two-dimensional array is hexagonal with $a = 620 \text{ \AA}$ and the light direction is coincident with the (11) direction of the two-dimensional arrays. The orientation of the two-dimensional array of microvilli relative to the light direction is shown in Fig. 3.

It is known from electron microscopy^{7,8} that the adjacent microvilli arrays of different retinula cells are approximately perpendicular to each other but it is not known to what extent this kind of packing is followed throughout the whole retina. If the X-ray specimen of squid retina contained only two orientations (which are perpendicular to each other) then the recording of the off-meridional reflections would be sensitive to the direction of the incident X-ray beam. But no such sensitivity has been recorded. The pinhole-collimated X-ray patterns indicate that the (11) direction is a rotation axis. Thus, although adjacent arrays may be perpendicular to each other^{7,8}, this kind of packing is not maintained over the whole retina.

A model for a single microvillus is a long thin cylinder where the wall is made up of a single membrane of width m . It is assumed that $m \sim 75 \text{ \AA}$ —that is, the microvillus membrane has the same thickness as the disk membrane¹⁻⁴—and that the outer surfaces of the cylinders are separated by a fluid layer about $5 \text{ \AA}$ wide. The single membrane is centred at a distance r from the axis of the cylinder and $r = 1/2 (620 - m - 5) \text{ \AA}$, that is, $r = 270 \text{ \AA}$. The Fourier transform of the membrane with respect to its centre is denoted by $M(R)$ where R is the reciprocal space coordinate. The Fourier transform of the two-dimensional array of microvilli is denoted by $T(R)$; and using X-ray diffraction theory appropriate to concentric membrane layers¹¹, $T(R)$ can be written

$$T(R) \sim 2\pi r J_0(2\pi r R) M(R) \Phi(R)$$

where J_0 is the zero-order Bessel function and Φ is the interference function for the two-dimensional array.

A test of the correctness of the above model can be made by comparing the calculated and observed intensities for the (10), (11) and (20) reflections. In the squid retina the two-dimensional arrays of microvilli are effectively rotated about the (11) direction. To refer intensities to an unrotated array the following corrections are made. The off-meridional reflections (01),(10) and (12),(21) are increased four to five times, while the off-meridional reflections (02),(20) and (13),(31) are increased eight to ten times. The X-ray intensities for

the first three reflections of the unrotated array have the following variation: $I(10) \gg I(20) > I(11)$. To calculate intensities from our model it is necessary to first estimate values of $M(R)$, the Fourier transform of the microvillus membrane. For the first two or three reflections which correspond to very small angles of diffraction, $M(R)$, can be approximated¹¹ by a membrane of constant density so that $M(R)$ is proportional to $(1/R) \sin(\pi m R)$ and where $m \sim 75 \text{ \AA}$. The $J_0(2\pi r R)$ variation is sensitive to the choice of r and we use $r = 270 \text{ \AA}$. By combining the $M(R)$ and $J_0(2\pi r R)$ variations, our model provides an intensity variation for the first three reflections, which is the same as recorded experimentally. Thus, the observed intensities of the first few reflections in the pinhole-collimated X-ray patterns are in agreement with the microvillus membrane having a thickness of about $75 \text{ \AA}$ and with the concentric membrane having a radius of about $270 \text{ \AA}$.

In summary, it has been possible to record X-ray diffraction from the photoreceptor structures of squid retina. So far, these X-ray patterns refer to fixed eyes. X-ray analysis of the pinhole-collimated patterns has provided information on the dimensions of the microvilli and on the molecular organisation of the microvilli in the distal segments of squid retina. Information on the internal structure of the microvillus membrane has not been obtained but this information may be forthcoming in future work provided that more reflections can be recorded in pinhole-collimated X-ray patterns.

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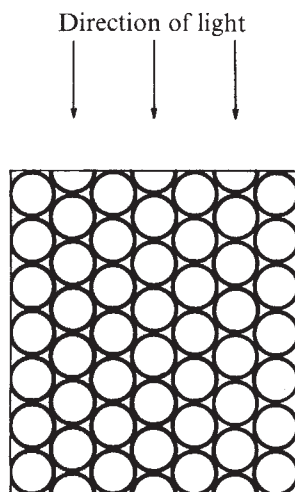
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Fig. 3 A drawing of a section through the two-dimensional hexagonal array of microvilli. The section contains the light direction and is perpendicular to the long axes of the microvilli cylinders.



Corrigendum

In the article "Intact 3' end of 16S rRNA is not required for specific mRNA binding" by J. V. Ravetch and K. S. Jakes (*Nature*, **262**, 150; 1976) the sentence at the beginning of paragraph 4 should read "The colicin-treated ribosomes used in the experiment shown in Fig. 1 were lowered in their ability to carry out f2 RNA-directed protein synthesis by 75%" and not as printed.

Errata

In the article "Constraints on the nature of the ancient lunar magnetic field" by J. N. Goswami (*Nature*, **261**, 675; 1976) two lines were omitted from the end of page 676. The sentence 'One of these . . . Fe-FeS . . .' should continue ' . . . melt a few hundred kilometres below the lunar surface, initiating such local fields' on the Moon, is yet to be verified.'

The title of the article by M. S. N. Carpenter and L. Civetta (*Nature*, **262**, 276; 1976) should read "Hercynian high pressure/low temperature metamorphism in the Ile de Groix Blueschists" and not as printed.

matters arising

Ionic clustering and collagen specificity

PIEZ and Torchia¹ agreed with us² that pairs of unlike charged residues in the collagen amino acid sequence are important for the assembly of molecules into fibrils. They described their use of three-dimensional molecular models to examine specific interactions and in doing so criticised our earlier one-dimensional treatment, in particular the dipole formalism used.

One-dimensional analyses of the collagen sequence proved fruitful in earlier work, when it was found that two-thirds of the charged amino acid residues behaved as if they were arranged in pairs of unlike charge^{3,4}. We simplified our calculations by treating these 100 paired charges as 50 dipoles; an approach commonly used as, for example, in the treatment of dielectric materials.

Contrary to the belief of Piez and Torchia, our aim was not to suggest a detailed interaction scheme of the type shown in their Fig. 1a; we were only suggesting an important role for the paired charges in specifying an inter-into fibrils. They described their use of molecular 1 D stagger. It is difficult to extrapolate this result to determine details of the arrangement of interacting charged residues in three dimensions. Nevertheless paired, unlike unpaired, charges are distributed in the collagen sequence in a manner which is intimately associated with the D stagger⁵. An understanding of this specificity in three dimensions, whether or not the concept of charge clustering is invoked, must await the outcome of a full three-dimensional analysis.

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PIEZ AND TORCHIA REPLY—We are pleased to read the statement of Doyle et al.¹ that we² inferred more from their dipole treatment³ than they meant us to. We would point out, however, that our Fig. 1a is essentially identical to their Fig. 1b. In any case, we seem to be agreed that charge groupings (pairs or clusters) are associated with molecular packing of collagen and that a three-dimensional analysis is needed to sort out exactly how they are associated.

A recent rereading of the early literature has reminded us that charge clustering is not a new idea. It was originally proposed by Hodge and Schmitt⁴ from electron microscopic studies of positively stained native collagen fibrils and SLS segments. We are, however, now able to treat the question at a much higher degree of resolution.

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Retinal sensitivity to short wavelength light

THE recent article¹ on potential hazards due to exposure of the primate retina to visible radiation of short wavelengths contains a number of conclusions which are open to argument.

The lesions are believed to be mediated essentially by (unspecified) photochemical mechanisms. The reciprocity law is not, however, obeyed in the data shown in Table 1, although both human rods and human cones² obey it in the timespan used by the authors.

The contribution of thermal effects is minimised even though the action spectrum bears a striking similarity to the absorption spectrum of melanin³.

The protection afforded by the crystalline lens is emphasised in so far as the retina is concerned, although the vitreous may be at risk in aphakic eyes^{4,5}.

The morphology of the lesion is virtually ignored, even though it has been successfully studied elsewhere⁶.

The authors write that a threshold lesion occurs when one views the Sun for not less than 100 s. In our view it should not be implied that a shorter period is safe in their experimental conditions.

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¹ Ham, W. T., Mueller, H. A., and Sliney, D. H., *Nature*, **260**, 153 (1976).

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HAM REPLIES—According to our best judgment, there are at least three types of radiation damage to the retina in the spectral range, near infrared to near ultraviolet (1,400-400 nm). These are: sonic transient or shock damage from picosecond to nanosecond pulses¹⁻³, thermal damage (independent to a first approximation of wavelength) from microsecond to second pulses^{4,5}, and photochemical damage from long term exposure (>1 s) to the shorter wavelengths in the visible spectrum (approximately 500-400 nm)⁶. There is no sharp transition between these three types of retinal damage, either from the standpoint of wavelength or exposure time. We believe that Marshall's report¹ of retinal damage belongs in the general category of thermal damage as outlined here.

In response to the specific comments of Marshall and Weale⁷ we offer the following: (1) Not even thermal lesions obey a reciprocity law⁴. There is no particular reason to believe that long term photic damage should obey a reciprocity relation and, as we pointed

out in the paper⁸, we do not know the relationship of rods and cones to the observed funduscopic pathology. (2) Thermal effects are minimised only for long term exposure to the shorter wavelengths in the visible spectrum where maximum temperatures in the retina do not exceed a few °C above ambient. The absorption spectrum of melanin would be expected to correspond to the action spectrum for thermal damage. Our action spectrum includes both thermal and photochemical damage and its relationship to the melanin absorption spectrum is suspect but should not be overlooked. (3) The hazard to the retina rather than the vitreous for aphakic eyes is emphasised because the vitreous does not absorb radiation appreciably between 300 and 1,400 nm (ref. 9). (4) The morphology of the lesion is not ignored but undetermined to date. Extensive efforts are under way, using both light and electron microscopy, to determine the nature of the lesion. The subtleties of these lesions bear no relationship to those reported by Marshall *et al.*⁸. Calculations show that the minimal irradiance on the retina in their experiments was greater by a factor of 10³ than those we reported⁸. In other words, Marshall *et al.*, were dealing with a totally different phenomenon, thermal injury, whereas we were seeking to characterise a long term photic damage mechanism or mechanisms in the retina.

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Exudate-eating and tree-gouging in marmosets

KINZEY *et al.*¹ discuss an unusual sap-eating behaviour in the pygmy marmoset (*Cebuella pygmaea*) of upper Amazonian South America. We have observed similar exudate-eating behaviour patterns in southern Brazilian marmosets of the genus *Callithrix* and would like to discuss briefly their significance.

Like *Cebuella*, *Callithrix* spp. are "short tusked": in other words, they have an unusual lower anterior denti-

tion in which the canines are short and the incisors long and roughly equal in length to the canines. The other two callitrichid genera, *Leontopithecus* and *Saguinus*, are "long tusked", with normal, long lower canines and short lower incisors. In *Callithrix* and *Cebuella*, the "short tusked" lower anterior dentition is apparently used as a gouge or scraper to perforate the bark and the superficial layer of the cambium of certain trees. When attacked in this manner, the trees exude gums and sap which are important food sources for the marmosets.

Free-living *Callithrix jacchus* have been observed gouging several species of trees (*Anacardium occidentale*, Anacardiaceae; *Tapirira guianensis*, Anacardiaceae; *Terminalia catappa*, Combretaceae) in the Brazilian states of Alagoas and Rio de Janeiro. Favourite trees are sometimes riddled with holes on all surfaces. Captive *Callithrix* (including *C. flaviceps*, *C. geoffroyi*, *C. jacchus* and *C. penicillata*) gouge holes in fresh branches and also in the dry wooden fixtures of their cages (Fig. 1). In addition to feeding on exudates that flow from holes in the fresh branches, captive (and probably also wild) *Callithrix* use the holes in both dry and fresh wood as foci for marking behaviour. High-ranking males and females frequently urinate in them and genital-rub in their vicinity. The gouging process in *Callithrix* is usually accomplished by anchoring the upper incisors on the substratum and scraping with the lower anterior teeth (Fig 1). In both *Cebuella* and *Callithrix*, wear patterns on the occlusal surface of the upper incisors indicate that they are

Fig. 1 Captive *Callithrix geoffroyi* gouging hole in dry branch in its cage. Note the positions of the upper and lower teeth.



used to hone the tips of the lower incisors (personal communication from R. D. Martin). Depending on their hardness, exudates are either licked up or collected with teeth. In contrast to *Cebuella* and *Callithrix*, the "long-tusked" *Leontopithecus* and *Saguinus* have never been observed gouging holes in wood, although they occasionally chew on strips of bark and feed on already-exuded gums if they happen to come across them.

An analysis of the gum of *Anacardium occidentale* gives an indication of the food value of exudates. The gum of this species is 84% carbohydrate and contains several minerals, including iron, aluminum, calcium, silicon, potassium and traces of magnesium and sodium³. These substances therefore provide small primates like *Callithrix* and *Cebuella* with a high energy food source not used to any great extent by larger primates and other potential competitors, and helps them to, at least in part, avoid competition for fruit, the major high energy food source for many tropical mammals and birds.

Sporadic exudate-eating from sites where insect infestation or mechanical damage has caused it to flow is a fairly common pattern in primates. The same can be said for occasional use of the teeth to pry, gouge, strip or break bark from trees. In addition, certain prosimians (for example, *Euticus elegantulus*, *Microcebus murinus* and *Galago senegalensis*) use their specialised "tooth combs" or "tooth scrapers", formed by the lower anterior dentitions, as scoops to collect gums from areas where they have already exuded (personal communication from R. D. Martin). But, the regular use of tree gouging specifically to elicit exudate flow has so far been found only in *Callithrix*, *Cebuella* and the Madagascan fork-marked dwarf lemur *Phanerfurfur*⁵ and seems to be rare not only among primates but among vertebrates in general.

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reviews

Meeting the challenge of astronomy

Paul Davies

Black Holes, Quasars and the Universe. By H. L. Shipman. Pp. 309. (Houghton Mifflin: Boston, Massachusetts, 1976.) \$12.95. Paperback edition available in UK (Houghton Mifflin: London, 1976.) £2.50.

THIS is an astronomy book written in the best Fred Hoyle tradition—informative without being boring, accurate without lacking excitement and comprehensible to a wide range of readership from the intelligent layman to the specialist. For the reader who wishes to actually learn some modern astronomy, as well as be entertained and intrigued, Dr Shipman's book is for them.

For some years modern topics like black holes and quasars have received only scant attention in the serious literature, and have been the subject of much sensationalistic, science-fiction-style speculation in popular books. The treatment presented here is hard-headed, down-to-earth and carefully factual. Nevertheless, this does not prevent the author from indulging his and our imagination in some of the possible far-reaching consequences of recent advances in theoretical and observational astronomy. But always Dr Shipman puts us back on the straight-and-narrow when the speculation becomes too divorced from the known facts. He continually draws a distinction between the model world of the theoriser, and the real world of telescopes and observations. Speculation is fun, and could be right—but remember it is only speculation.

Within this commendable framework, the author then proceeds to review all the latest exciting developments in astronomy and cosmology over the past decade or so. The text is crammed full of interesting facts, but the engaging style prevents the reader from tiring with an information overload. The book is divided into three sections. The first deals with black holes—both the underlying theory and the X-ray sources, which are observational contenders for real black holes. Dr Shipman emphasises how theory has far outstripped observation in this topic. At the end of this section a cautionary summary distinguishes the different levels of discussion presented

—the strictly factual, the well-founded theory, and the wilder speculations. A clear distinction between them is urged on the reader.

Section II is devoted to quasars, a field in which observations are numerous and theoretical understanding minimal. Many technical details of modern astronomical procedures are clearly and simply explained. No discernable theoretical axe is ground by the author. The more bizarre explanations of quasar redshifts are carefully separated from the rest.

The final section deals with cosmology. The treatment is straightforward and up-to-date, although a little brief.

The presentation of the book is excellent. A preparatory section explains in simple language some of the ele-

mentary concepts needed for the rest of the book, including a fine discussion of the role of theoretical models and the way in which scientific progress takes place. A glossary and bibliography are also given.

The author's style is brilliant. Direct, simple, clear and informative. This is a no-nonsense book which meets the challenge of modern ideas without losing contact with reality. It is admirably suited to introductory astronomy courses, and can be easily read and enjoyed by science specialists and non-scientists alike. It is certainly one of the best science books I have read for a long time. □

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Galaxy Formation: A Personal View. By John Gribbin. Pp. viii+79. (Macmillan: London and Basingstoke, May 1976.) Hardcover £5.95; papercover £2.95.

ONE of the key observational principles in Astronomy is that which links lifetime with number of observable objects. A star, for example, spends a large fraction of its lifetime as a main sequence object, and a much shorter time as a protostar, so that we can observe relatively far fewer protostars at any epoch. An often-used piece of astronomical method is the use of statistics to overcome our inability to experiment, and above all to experiment in a controlled way. Theories of stellar evolution involving chains of nuclear processes with different probabilities are tested against the large number of available stellar spectra in which the end products of the reactions are exhibited. Stellar evolution theory is by now one of the most firmly based branches of Astrophysics.

The situation with galaxy evolution theory is far less developed. Partly this is because the formation phase, as with stars, is likely to be only a restricted fraction of the galaxy lifetime, so that protogalaxies would be inherently few. Partly it is because galaxies are distant and composite objects, whose structure and chemical composition are hard to

determine finely. Above all it is, as John Gribbin has correctly stressed in his book, because galaxy formation and evolution are so closely bound to the macroscopic properties of the observable universe. For galaxies the lifetime-frequency link is useless as an observing tool until one has answered the question "Were all galaxies formed at a single epoch, or is galaxy formation continuous?"—or some more qualified version of the question. If at a single epoch, the key to understanding is to observe at distances currently not possible to reach—seeing the universe near to the origin of its current expansion phase, when pregalactic objects may have existed—whereas continuous creation of galaxies is hard to sustain either observationally or on cosmological theory.

John Gribbin has done a useful job of gathering into a short and readable space much of the current thinking about galaxy formation. He rejects the traditional view of galaxies forming by gravitational instability within gas clouds, because there seems to be no way of forming entities exclusively within the observed mass range. Ambartsumian has tried to overcome this problem by postulating that the fragmentation process is still going on, since clusters of galaxies may well not be gravitationally bound, and galaxies themselves are in states of central ac-

tivity which may amount to disruption. It is difficult, however, to see how the energy to feed the disruption, or the spin-up of a galaxy, can be produced quickly enough in normal galaxies, with their observed central densities. A hypothesis of Layzer that galaxies form by agglomeration of matter, and not by fragmentation, falls down, as Gribbin notes, because the material which makes planets seem observationally to have come out of stars, whereas during agglomeration planets would form first.

Having thus surveyed earlier work, the author turns to what he sees as a synthesis combining some attractive features of the continuous creation cosmology and currently accepted views of a singular explosive origin for the universe. Essentially, galaxies are little pockets of the universe having galactic mass whose cosmogonic expansion has been somehow delayed.

Since each pocket starts close to its Schwarzschild radius, there is enough free energy available to bring about high energy outbursts typical of quasars. A quiet galaxy is formed later in the evolutionary sequence, either as an elliptical, when little material surrounds the initial pocket, or a spiral, if it is surrounded by gas and dust. As a picture this raises as many problems as it solves. Above all, the basis for postulation is entirely arbitrary on the present state of the evidence, at least as arbitrary as alternative hypotheses in predicting galaxies with an observed mass distribution. This is treated very lightly as a 'boundary value problem' dependent on the initial conditions of the universe at the time of the big bang, but this approach is without much predictive power, and the weaker for that.

On the cover John Gribbin's book

is described as "suitable for the second year student of physics" as indeed it is in some ways. Most physicists, as opposed to astrophysicists, will find the abundance of hand-waving arguments and the tendency to cull formalism from the air thoroughly infuriating, but in a topic as variegated and alive as this, it is one of the effective ways of introducing ideas seriously, without the dead weight of rigour. The author is aware of pitfalls, and not to be strongly faulted here. As a useful summary of a tangled topic this volume should find its place on many a professional's bookshelf, and on those of students who can afford it.

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Mössbauer again

Principles of Mössbauer Spectroscopy. (Studies in Chemical Physics.) By T. C. Gibb. Pp. 254. (Chapman and Hall: London, Halsted: New York, March 1976.) £9.

YET another book on the principles of Mössbauer spectroscopy! The author in fact lists seven similar texts in his bibliography although admittedly only three of these cover the same field of chemical applications.

The basic format consists of two chapters concerned with the general principles of the technique followed by nine chapters in which the applications are illustrated with some 200 references to published work. The main body of the book is made up of three chapters in which the use of the measured hyperfine interactions between the nucleus and its electronic surroundings is discussed, both in the study of chemical bonding, and also in structure determinations of molecular complexes. Here, as elsewhere, the examples are not restricted to the well studied isotope ^{57}Fe alone, but cover the use of tin, antimony, iodine, ruthenium and iridium isotopes as well as several lanthanides and actinides. These chapters are followed by one in which the effects on the Mössbauer resonance of the dynamic processes involving lattice vibrations, atomic diffusion and spin relaxation are discussed; and one concerned with the structures and magnetic properties of spinels. The use of Mössbauer isotopes as impurity probes is illustrated including a mention of the novel experiments to measure the hyperfine interactions at an isolated Mössbauer atom obtained by condensation with an inert gas. Finally there are chapters outlining studies of the

effects of atomic environment in alloys, analytical applications and the properties of iron atoms in protein molecules.

Throughout the style is clear and readable. No attempt is made to discuss the instrumentation required and the use of mathematics is kept to a minimum; but there is nevertheless a lot of information packed in some 200

pages. There are similar texts available but this present volume probably covers a wider range of measurements and should prove a useful introduction for the student. **G. Longworth**

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Pharmacological marriage

Behavioural Pharmacology. By Susan D. Iversen and Leslie L. Iversen. Pp. xiv+310. (Oxford University: New York and London, 1975.) £2.75 paper; £5.50 boards.

THE Iversens' marriage of neuro- and psychopharmacology has produced a remarkably sophisticated, yet readable, text on behavioural pharmacology. The text is self-contained and appropriate for advanced undergraduate or first-year graduate students. A wide spectrum of drugs are included: amphetamines, barbiturates, hormones, anti-depressants, opiates, benzodiazepines, phenothiazines and butyrophenones, hallucinogens and cannabis.

The neurochemical chapters cover broadly but carefully the basic enzymology and pharmacology of all the neurotransmitters. Modern techniques, such as histofluorescence and iontophoresis, are introduced; and methodological problems are discussed, such as avoiding the blood-brain barrier in pharmacological experiments.

The behavioural chapters are written from the perspective of 'descriptive behaviourism', with emphasis on

schedules of reinforcement. Coverage is sufficiently critical and thorough to be useful for research workers as well as students. Distinctive profiles emerge for drugs such as benzodiazepines (release of behaviour suppressed by punishment) and phenothiazines (decrease of discriminative stimulus control). Ethologically-minded readers may be distressed to see changes in rate of responding after increases or decreases in the size of reinforcement offered as a model for elation and depression, whereas depressive states induced in primates by separation are dismissed as "a difficult condition to induce experimentally". There are reliable, quantitative paradigms, on which schizophrenic patients are known to differ from normals. One of them, the 'continuous performance test' of Kornetsky, has an animal version, but others—such as the series of reaction time paradigms developed by Shalowsky and his school—have not been reproduced in animals. One might hope that a literature of this kind would develop sufficiently to be included in a second edition of this excellent text.

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Archaeological classic

The Earlier Stone Age Settlement of Scandinavia. By Grahame Clark. Pp. xxv+282. (Cambridge University: London, January 1975.) £7.50; \$22.50.

THE appearance of this book is a major archaeological event. As the list of 'Works cited in the text' shows, Professor Clark has devoted much of his academic life to the study of the economic basis of Prehistoric Europe, and in particular the material from Scandinavia. This book typifies his distinctive approach, and is essentially a new version of *The Mesolithic Settlement of Northern Europe* published in 1936. It is, we are told, to be followed by a second volume on aspects of the Later Stone Age. As far as the present volume is concerned, Clark's frank admission that "when a book is thirty-eight years old . . . it is unlikely to be a useful vehicle for new information" has meant that the 1936 book has been left behind, and we now have a new book on new lines. It would be presumptuous for a regional rather than period specialist to assess in detail the academic quality of the work, but it is perfectly clear that Professor Clark's self-imposed task of a new synthesis has provided us with a modern classic of archaeological literature.

The book begins with discussions of 'Some basic concepts' such as eco-systems, subsistence and seasonality, and so forth. It leads naturally to the theme of 'Habitat and Biome', and, although a 'Chronological framework' appears at the beginning of each of the following four chapters, essentially they are seen from that economic viewpoint which Professor Clark has championed. Instead of chapter headings such as 'The Tanged-Point Cultures' and 'The Axe Cultures of the Lowland Forest Area', we now have four chapters on the 'Late-glacial Settlement of Denmark/Scania', 'Early Post-glacial Settlement in South Scandinavia', 'Atlantic settlement in south Scandinavia', and 'The older Stone Age Colonisation of the Fenno-Scandian Shield'. Instead of the chapter on 'The Art of the Maglemose Culture', 'psychic needs' (that is, disposal of the dead, personal adornment, and symbolic art) now make their appearance in appropriate places in all four chapters, along with technology and subsistence. The 'General Summary and Retrospect' has disappeared, and, although one might sometimes wish for the threads to be drawn together more, in fact this simply acts as an incentive to re-read the sections containing the basic data.

This work is both erudite and clear, likely to become a standard text immediately, and remain so for as long as the original book has been one. Students and specialists alike will be grateful for the five lists of objects and faunal assemblages printed as appendices, and also the seventeen pages of bibliographical references at the end. Nineteen tables and sixteen maps serve to present effectively in visual terms much vital information, and there are sixty-three figures in the text. It is slightly disconcerting to find considerable variation from one drawing to the next, and sometimes the reproduction leaves something to be desired. The plates also inform the text adequately, although here, more obviously in one or two cases the reproductions could certainly be better. In general, however, the book is well-produced, and the Syndics of the Cambridge University Press are to be congratulated on persuading Professor Clark to traverse this ground again. Volume two is eagerly awaited.

C. D. Morris

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Global aspects of primary production

Primary Productivity of the Biosphere. (Ecological Studies: Analysis and Synthesis, Vol. 14.) Edited by Helmut Lieth and Robert H. Whittaker. Pp. vi+339. (Springer: London and New York; Chapman and Hall: London, February 1976.) £14.50.

THE International Biological Program (IBP) has generated a wealth of publications concerning primary productivity, many of which are scattered through the literature. Several attempts have been made over the past few years to collate information, particularly that concerning the methodology involved in measuring primary production. These have taken the form of handbooks relating to specific vegetation types and symposium volumes of collected papers. The time is evidently ripe for the publication of a general review of the state of knowledge concerning global aspects of primary production so that some of the scattered data derived from IBP projects can be made easily available to students and other interested parties. The book under review here attempts just this task.

The book falls into four sections, a historical introduction, methods used in production measurement (almost half of the book), an assessment of the available information derived from production studies, and some thoughts on further research developments stemming from these data. The third section is likely to be the most useful to a wide spectrum of ecologists, since a very large volume of literature is reviewed and data is tabulated in a form which enables easy reference. Marine, freshwater and terrestrial biomes are covered and Helmut Lieth explains at some length his widely used models of global productivity patterns.

The authors are undoubtedly right in devoting a large section of the book to a discussion of methods. The estimation of productivity, whatever method is used, involves a series of successive estimations and the reliability of the final figures quoted can only be assessed if the nature of these approximations is appreciated. This reasoning must lie behind the presentation of these methods, for they are dealt with too briefly to be of value as laboratory or field guides to productivity measurements. More detailed accounts are easily available elsewhere, with the possible exception of the regional assessment of primary productivity and the compilation of productivity maps.

Many of the previously published papers and symposium volumes on the subject of primary productivity leave one with the impression that the measurement of this process is an end in itself. Lieth and Whittaker's book goes beyond this, and constantly draws attention to the importance of the measurements presented in relation to broader issues in theoretical ecology and in the study of man's pressures on the environment. These themes, which underlie the whole book finally emerge in an overt manner in the final section on the utilisation of the knowledge of primary production. Here such issues as the relationship between productivity and species diversity and the carrying capacity of the world for man are discussed.

The book sets out to synthesise modern knowledge and to make clear the implications of this knowledge as far as man and the biosphere are concerned. It succeeds admirably in these aims and the enthusiasm of the writers, which emerges from between the lines, will undoubtedly attract many students into this area of ecology.

Peter D. Moore

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obituary

David Clarke, who died in Cambridge on June 28, after a short illness, was a rare, almost unique figure in the academic world: he was a theoretical archaeologist. At the age of only 38 he had already established an international reputation, largely as a result of the massive volume entitled *Analytical Archaeology* (1972) and the volume of papers which he edited as *Models in Archaeology* (1972). Behind both of these lay his substantial research on late neolithic pottery in Britain, in which he had pioneered many revolutionary quantitative techniques.

It was as a result of this initial interest in classification that he became aware that many of the problems of archaeology had already been partly solved in neighbouring subjects. The work of Sokal and Sneath in numerical taxonomy provided an obvious parallel, while the quantitative revolution in geography—exemplified in Haggett's *Locational Analysis in Human Geography*—suggested exciting new possibilities for archaeology.

These developments stimulated him to attempt, in a general investigation of the techniques and procedures of archaeology, to define the unique qualities required of a subject whose raw material is the oblique record of two million years of hominid behaviour. It was his objective to trans-

form it from a body of partially synthesised knowledge and recognised but undefined procedures, into an explicit discipline for the investigation of the pre-literate past. This he did, not by attempting to force archaeology into a positivist hypothetico-deductive strait-jacket, in the manner of the American New Archaeology, but by an investigation of the kinds of model which had been, or could, potentially, be used in archaeology. These ranged from the abstract treatment of systems analysis to the carefully calibrated ethnographic comparisons with which he illuminated the patterns of prehistoric material culture. In all these his writing was marked by brilliant insights and the application of a fertile imagination. It was his aim to make archaeology a human science, but with unique opportunities for the study of particular scales and dimensions of behaviour, notably of long-term processes of change.

As he himself anticipated, his writing evoked a wide range of responses. Especially in America and Scandinavia it was eagerly taken up and soon became required reading for undergraduates. At his own university, Cambridge, he became involved in a productive dialogue with other viewpoints and emphases, to the benefit of both. In the rest of Britain, however,

his work was treated with reserve, verging on incomprehension: the enthusiasm of his written style, with its dense noun-clusters interspersed with vivid and striking metaphors and examples from an enormous range of reading, both within archaeology and outside, was the delight of the imaginative and the despair of the pedantic.

But a bare description of the aims of his written work gives only a partial view. It was his intuitive feel for the evidence and the questions which could be asked of it, as well as his infectious enthusiasm, which made him so impressive in discussion and as a teacher. Those who knew only Clarke the polemical author were charmed to discover a most unpretentious person whose interest both in the subject and those who wished to learn about it was most evidently expressed in the circle of students which gathered about him.

He combined an intellectual authority with a readiness to see other points of view and subsume them in his own. Always willing to give his time to those who wanted it, he was a friend and adviser to many beyond his own students. Both at a personal level, and as an intellectual discipline, archaeology is impoverished by his death.

Andrew Sherratt

announcements

Meetings

August 19–28, **Atmospheric Radiation**, Garmisch-Patenkirchen, FRG (The Secretariat, WMO, Geneva, Switzerland).

September 7–10, **Gas Discharges**, Swansea (IEE Conference Department, Savoy Place, London WC2R 0BL, UK).

September 25, **Short-Period Weather Forecasting**, London (The Executive Secretary, Royal Meteorological Society, James Glaisher House, Grenville Place, Bracknell, Berks. RG12 1BX, UK).

September 26–29, **Annual Fall Meeting of the American Oil Chemists' Society**, Chicago (AOCS, 508 S. Sixth Street, Champaign, Illinois 61820).

October 3–7, **Tumour Viruses**, Grindel-

wald, Switzerland (Xth European Tumour Virus Group Meeting, Secretariat, Department of Molecular Biology, Swiss Institute for Experimental Cancer Research, Bugnon 21, 1011 Lausanne, Switzerland).

October 12–15, **Electrical Phenomena at Membrane Level**, Saclay, France (Dr C. Troyanowsky, General Secretary, Société de Chimie physique (29th International Meeting), 10, rue Vauquelin, 75231 Paris, Cedex 05, France).

October 14–16, **RRIM Planters' Conference** (Chairman, Organising Committee, RRIM Planters' Conference 1976, Rubber Research Institute of Malaysia, PO Box 150, Kuala Lumpur 01-02, Malaysia).

October 17–22, **Water Pollution Research**, Sydney, Australia (Dr S. H. Jenkins, International Association on Water Pollution Research, Headington Hill Hall, Oxford OX3 0BW, UK).

October 25–29, **Medical Radionuclide Imaging**, Los Angeles (L. J. Johansson, Applications Section, International Atomic Energy Agency, PO Box 590, A-1011 Vienna, Austria).

October 26–28, **Experimental Use of Algal Cultures in Limnology**, Sandefjord, Norway (Knut Pedersen, Norwegian Institute for Water Research, PO Box 333, Blindern, N- Oslo 3, Norway).

November 8–9, **Genetic Mechanisms of Sexual Development**, Albany, New York (H. Lawrence Vallet, M.D., Symposium, Room 572, Empire State Plaza Tower Building, Albany, New York 12237).

December 5–7, **Freight Pipelines**, Washington, D.C. (Prof. I. Zandi, Room 113A Towne Building/D3, University of Pennsylvania, Philadelphia 19174).